Fig. 1

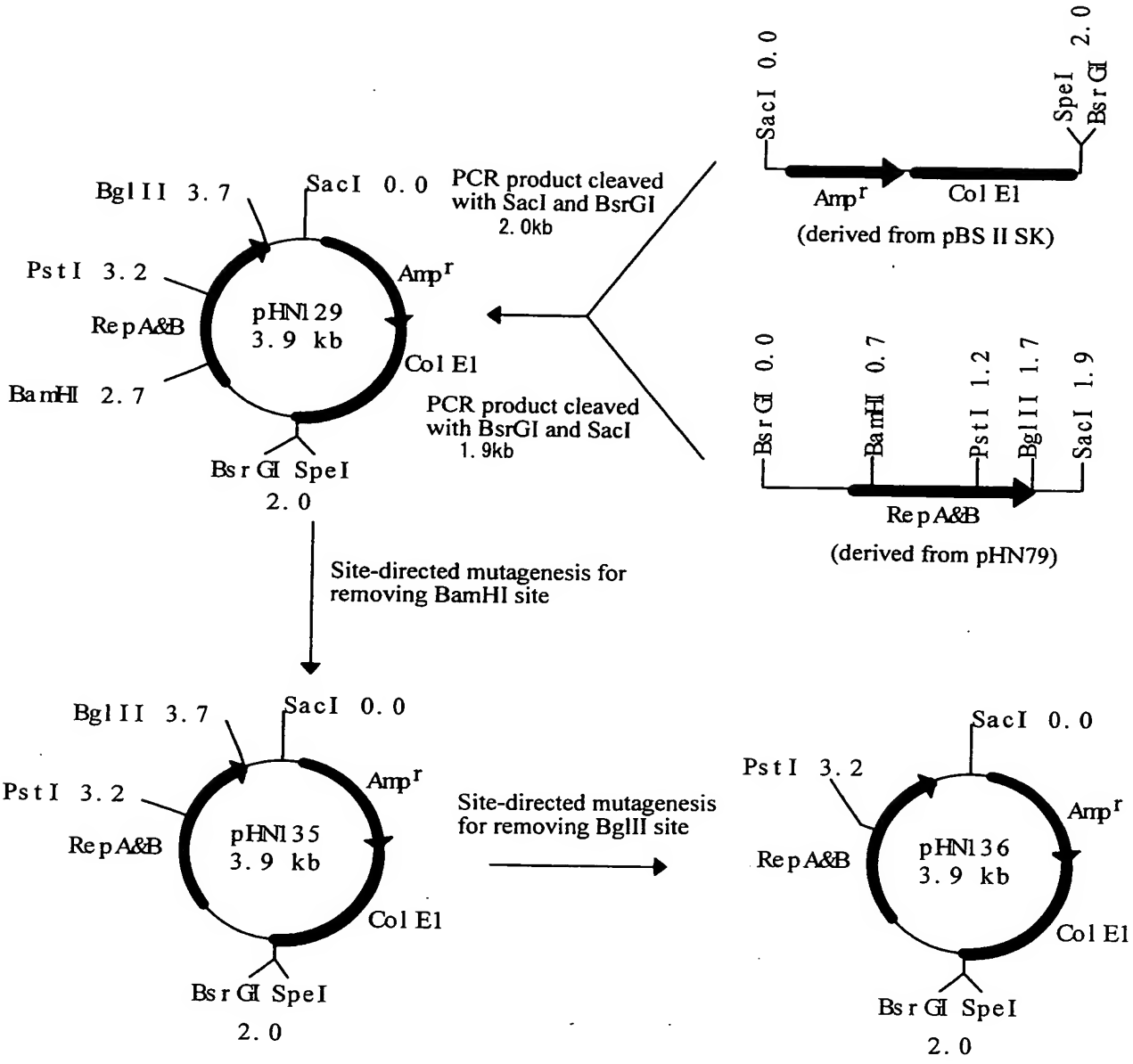
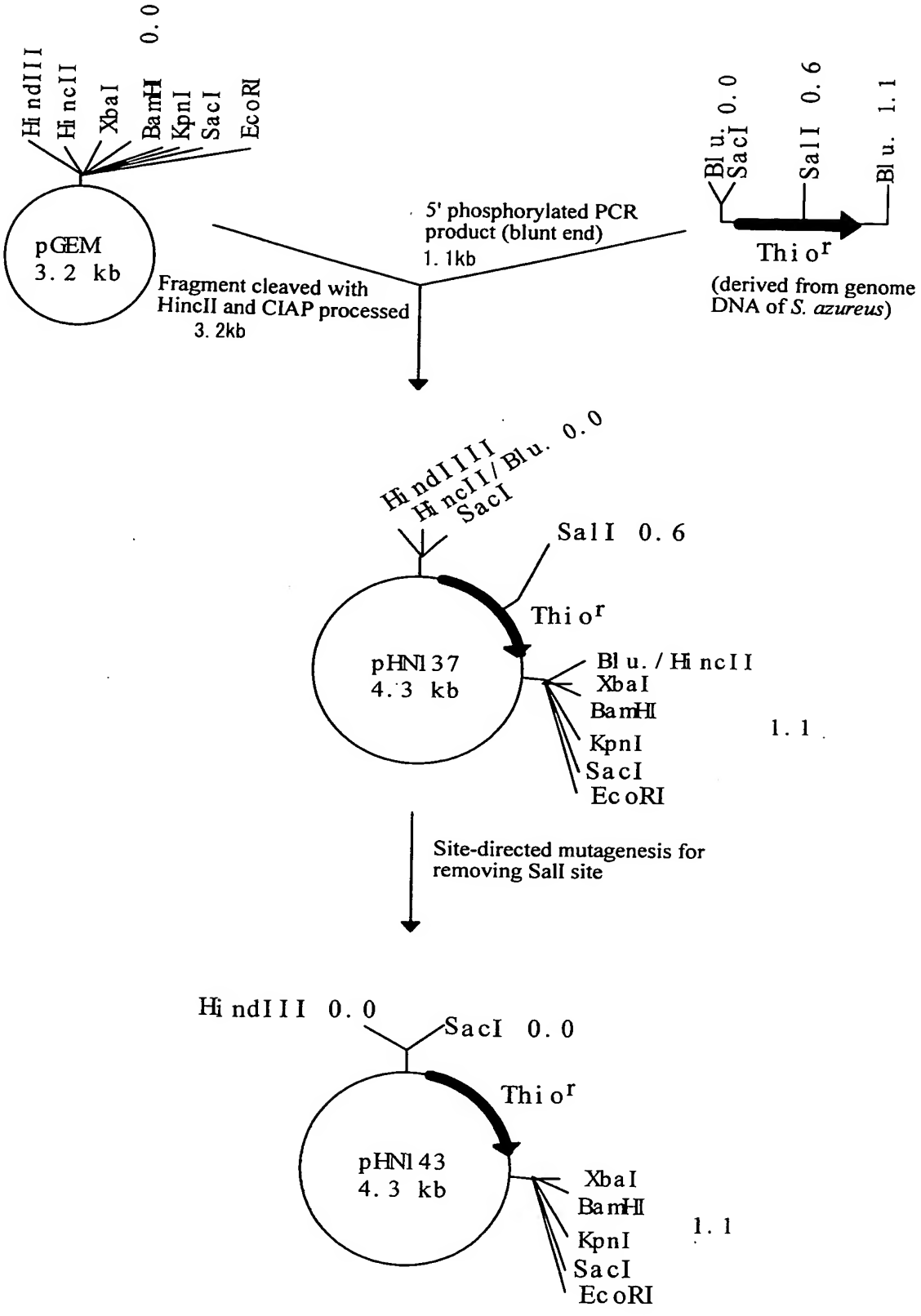

SacI 0.0
SpeI 2.0
BsrGI
Ampr
Col E1
(derived from pBS II SK)
PCR product cleaved with SacI and BsrGI
2.0kb
PCR product cleaved with BsrGI and SacI
1.9kb
BglII 3.7
SacI 0.0
PstI 3.2
RepA&B
BamHI 2.7
pHN129
3.9 kb
Ampr
Col E1
BsrGI SpeI
2.0
BsrGI 0.0
BamHI 0.7
PstI 1.2
BglII 1.7
SacI 1.9
RepA&B
(derived from pHN79)
Site-directed mutagenesis for removing BamHI site
BglII 3.7
SacI 0.0
PstI 3.2
RepA&B
pHN135
3.9 kb
Ampr
Col E1
BsrGI SpeI
2.0
Site-directed mutagenesis for removing BglII site
SacI 0.0
PstI 3.2
RepA&B
pHN136
3.9 kb
Ampr
Col E1
BsrGI SpeI
2.0

Fig. 2


HindIII
HincII
XbaI
BamHI
KpnI
SacI
EcoRI
0.0
pGEM
3.2 kb
Fragment cleaved with
HincII and CIAP processed
3.2kb
5' phosphorylated PCR
product (blunt end)
1.1kb
Blu.
SacI
0.0
SalI 0.6
Blu. 1.1
Thio^r
(derived from genome
DNA of S. azureus)
HindIII
HincII/Blu.
SacI
0.0
SalI 0.6
Thio^r
pHN137
4.3 kb
Blu./HincII
XbaI
BamHI
KpnI
SacI
EcoRI
1.1
Site-directed mutagenesis for
removing SalI site
HindIII 0.0
SacI 0.0
Thio^r
pHN143
4.3 kb
XbaI
BamHI
KpnI
SacI
EcoRI
1.1

Title: PROCESS FOR PRODUCING RECOMBINANT PROTEIN IN BACTERIUM BELONGING TO THE GENUS RHODOCOCCUS
Inventor(s): Nobutaka NAKASHIMA, et al.
DOCKET NO.: 081356-0253

Fig. 3

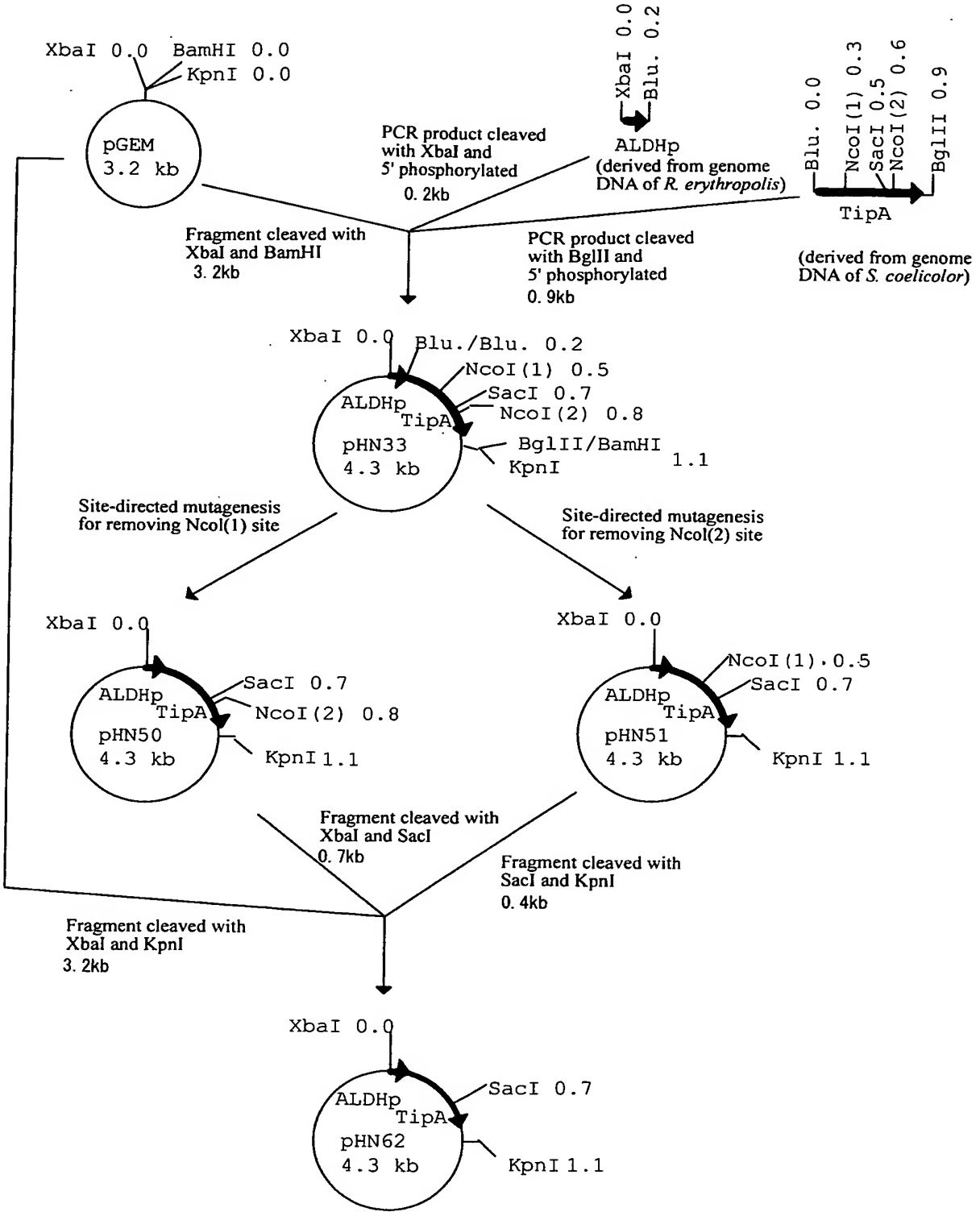
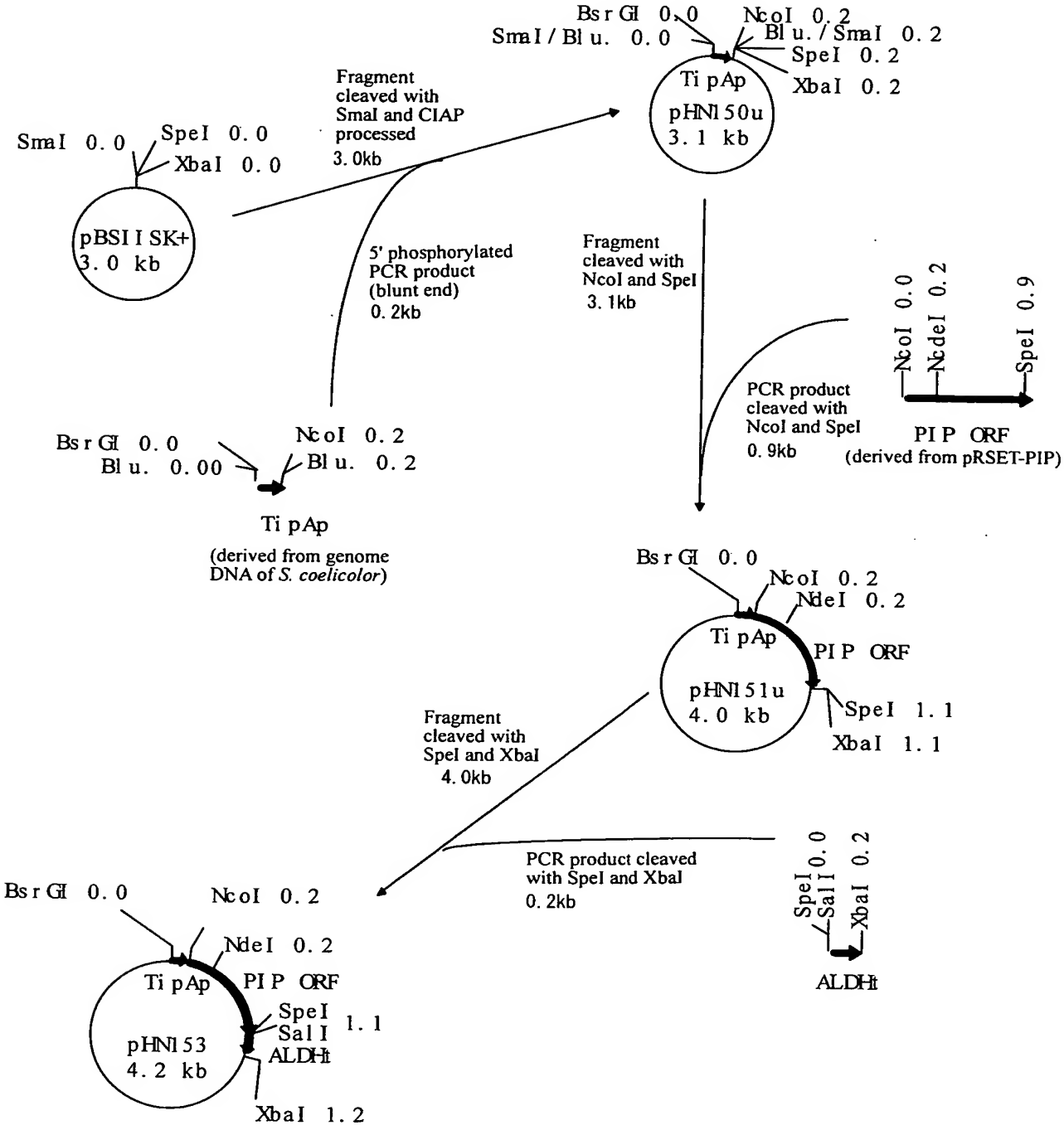


Fig. 4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NcoI 0.2
SmaI/Blu. 0.0
Blu./SmaI 0.2
SpeI 0.2
XbaI 0.2
TipAp
pHN150u
3.1 kb
Fragment cleaved with SmaI and CIAP processed
3.0kb
SmaI 0.0
SpeI 0.0
XbaI 0.0
pBSII SK+
3.0 kb
5' phosphorylated PCR product (blunt end)
0.2kb
Fragment cleaved with NcoI and SpeI
3.1kb
NcoI 0.0
NdeI 0.2
SpeI 0.9
PCR product cleaved with NcoI and SpeI
0.9kb
PIP ORF
(derived from pRSET-PIP)
BsrGI 0.0
Blu. 0.00
NcoI 0.2
Blu. 0.2
TipAp
(derived from genome DNA of S. coelicolor)
BsrGI 0.0
NcoI 0.2
NdeI 0.2
TipAp
PIP ORF
pHN151u
4.0 kb
SpeI 1.1
XbaI 1.1
Fragment cleaved with SpeI and XbaI
4.0kb
PCR product cleaved with SpeI and XbaI
0.2kb
SpeI 0.0
SalI 0.2
XbaI
ALDHt
BsrGI 0.0
NcoI 0.2
NdeI 0.2
TipAp
PIP ORF
SpeI
SalI 1.1
pHN153
4.2 kb
ALDHt
XbaI 1.2

Fig. 5

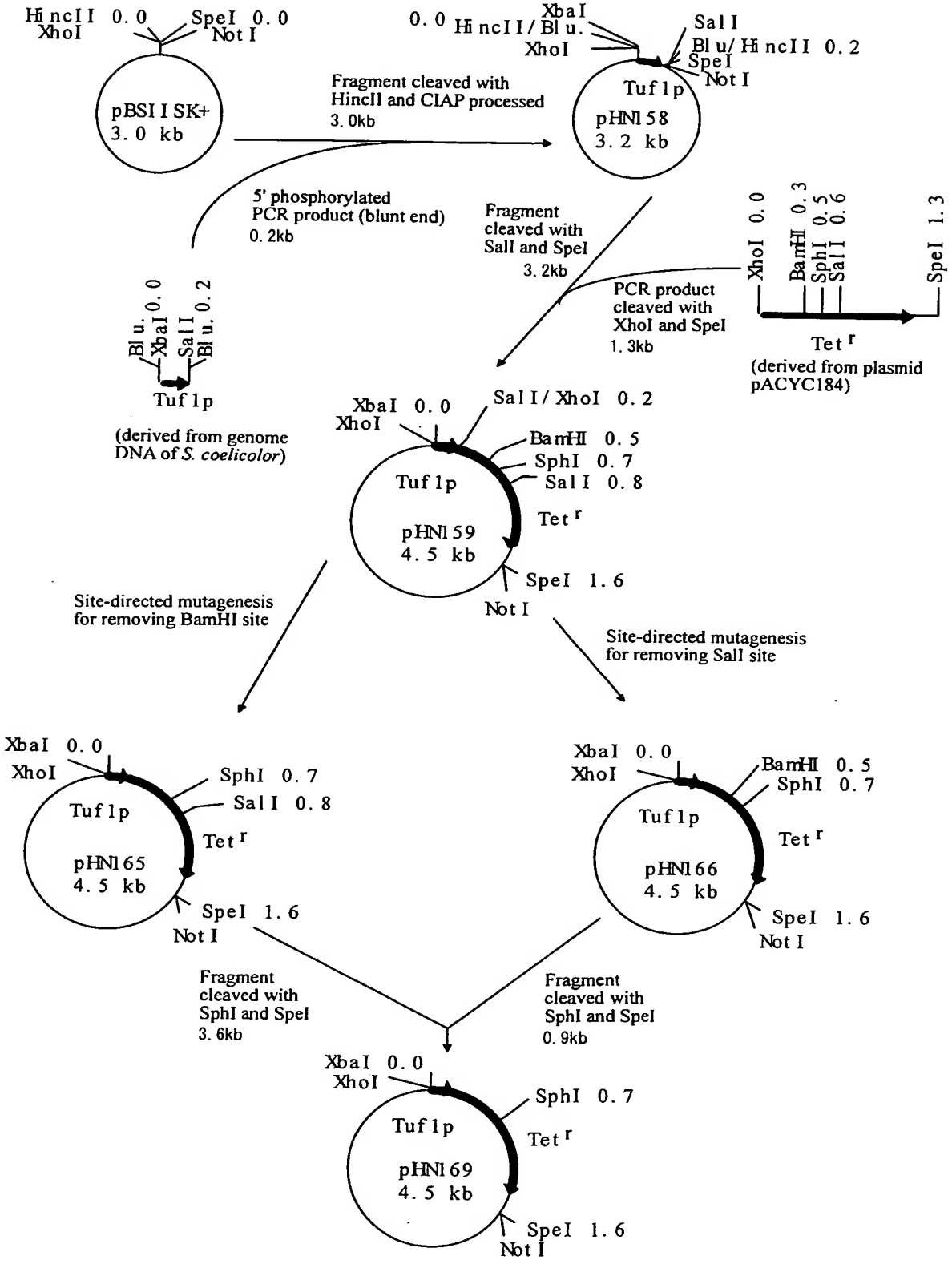

HincII 0.0
XhoI
SpeI 0.0
Not I
pBSII SK+
3.0 kb
Fragment cleaved with HincII and CIAP processed
3.0kb
0.0 HincII/Blu.
XbaI
XhoI
SalI
Blu/HincII 0.2
SpeI
Not I
Tuf1p
pHN158
3.2 kb
5' phosphorylated PCR product (blunt end)
0.2kb
Fragment cleaved with SalI and SpeI
3.2kb
PCR product cleaved with XhoI and SpeI
1.3kb
XhoI 0.0
BamHI 0.3
SphI 0.5
SalI 0.6
SpeI 1.3
Tet r
(derived from plasmid pACYC184)
Blu. XbaI 0.0
SalI
Blu. 0.2
Tuf1p
(derived from genome DNA of S. coelicolor)
XbaI 0.0
XhoI
SalI/XhoI 0.2
BamHI 0.5
SphI 0.7
SalI 0.8
Tet r
Tuf1p
pHN159
4.5 kb
SpeI 1.6
Not I
Site-directed mutagenesis for removing BamHI site
Site-directed mutagenesis for removing SalI site
XbaI 0.0
XhoI
SphI 0.7
SalI 0.8
Tet r
Tuf1p
pHN165
4.5 kb
SpeI 1.6
Not I
XbaI 0.0
XhoI
BamHI 0.5
SphI 0.7
Tet r
Tuf1p
pHN166
4.5 kb
SpeI 1.6
Not I
Fragment cleaved with SphI and SpeI
3.6kb
Fragment cleaved with SphI and SpeI
0.9kb
XbaI 0.0
XhoI
SphI 0.7
Tet r
Tuf1p
pHN169
4.5 kb
SpeI 1.6
Not I

Fig. 6

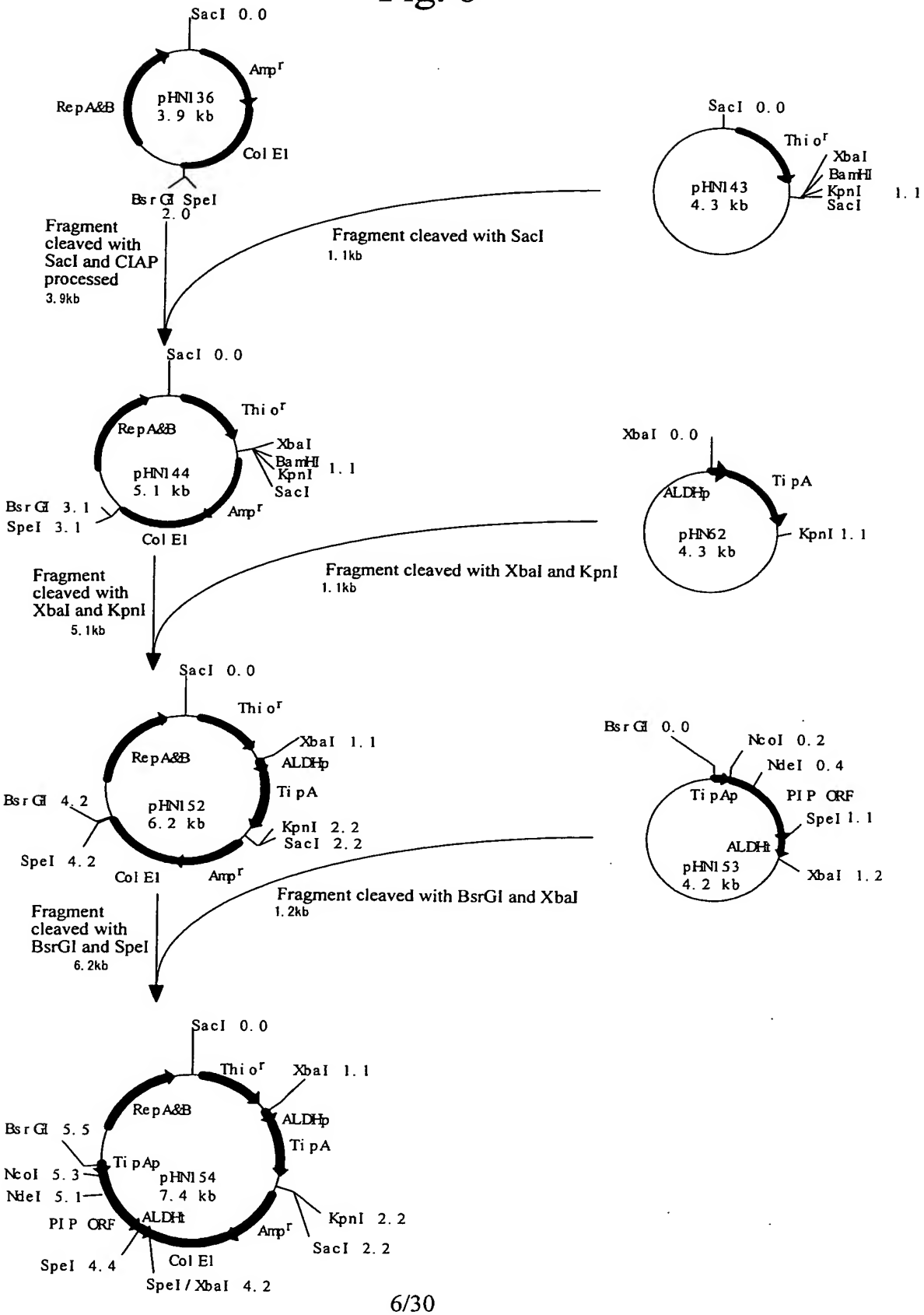

SacI 0.0
Amp^r
RepA&B
pHN136
3.9 kb
ColE1
BsrGI SpeI
2.0
Fragment cleaved with SacI and CIAP processed
3.9kb
SacI 0.0
Thio^r
XbaI
BamHI
KpnI
SacI
1.1
pHN143
4.3 kb
Fragment cleaved with SacI
1.1kb
SacI 0.0
Thio^r
RepA&B
XbaI
BamHI
KpnI
SacI
1.1
pHN144
5.1 kb
BsrGI 3.1
SpeI 3.1
Amp^r
ColE1
Fragment cleaved with XbaI and KpnI
5.1kb
XbaI 0.0
TipA
ALDHp
KpnI 1.1
pHN62
4.3 kb
Fragment cleaved with XbaI and KpnI
1.1kb
SacI 0.0
Thio^r
RepA&B
XbaI 1.1
ALDHp
TipA
KpnI 2.2
SacI 2.2
BsrGI 4.2
SpeI 4.2
pHN152
6.2 kb
ColE1
Amp^r
Fragment cleaved with BsrGI and SpeI
6.2kb
BsrGI 0.0
NcoI 0.2
NdeI 0.4
TipAp
PIP ORF
SpeI 1.1
ALDHt
XbaI 1.2
pHN153
4.2 kb
Fragment cleaved with BsrGI and XbaI
1.2kb
SacI 0.0
Thio^r
XbaI 1.1
RepA&B
ALDHp
TipA
BsrGI 5.5
TipAp
NcoI 5.3
NdeI 5.1
pHN154
7.4 kb
PIP ORF
ALDHt
Amp^r
KpnI 2.2
SacI 2.2
SpeI 4.4
ColE1
SpeI/XbaI 4.2

Fig. 6 (continued)

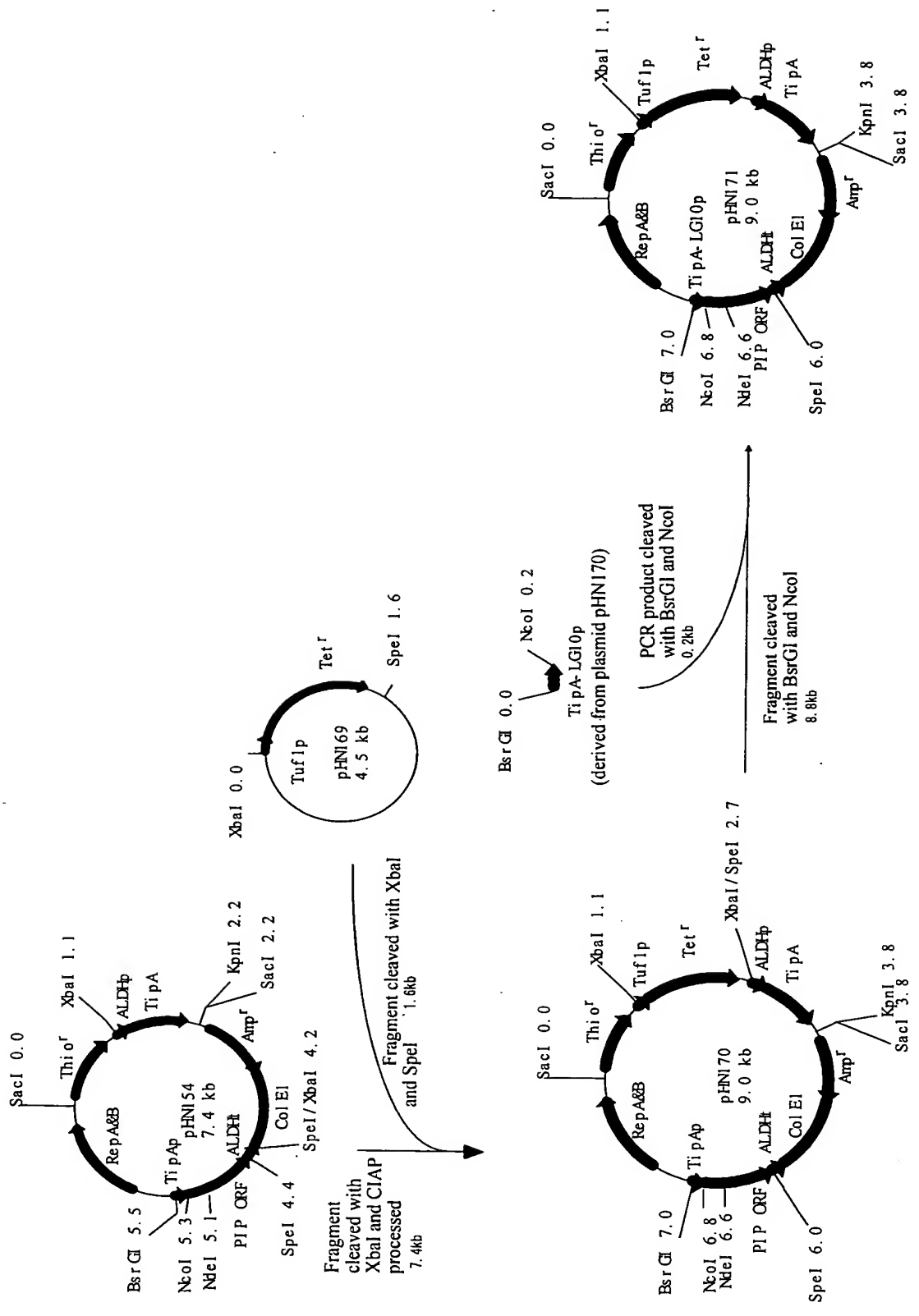
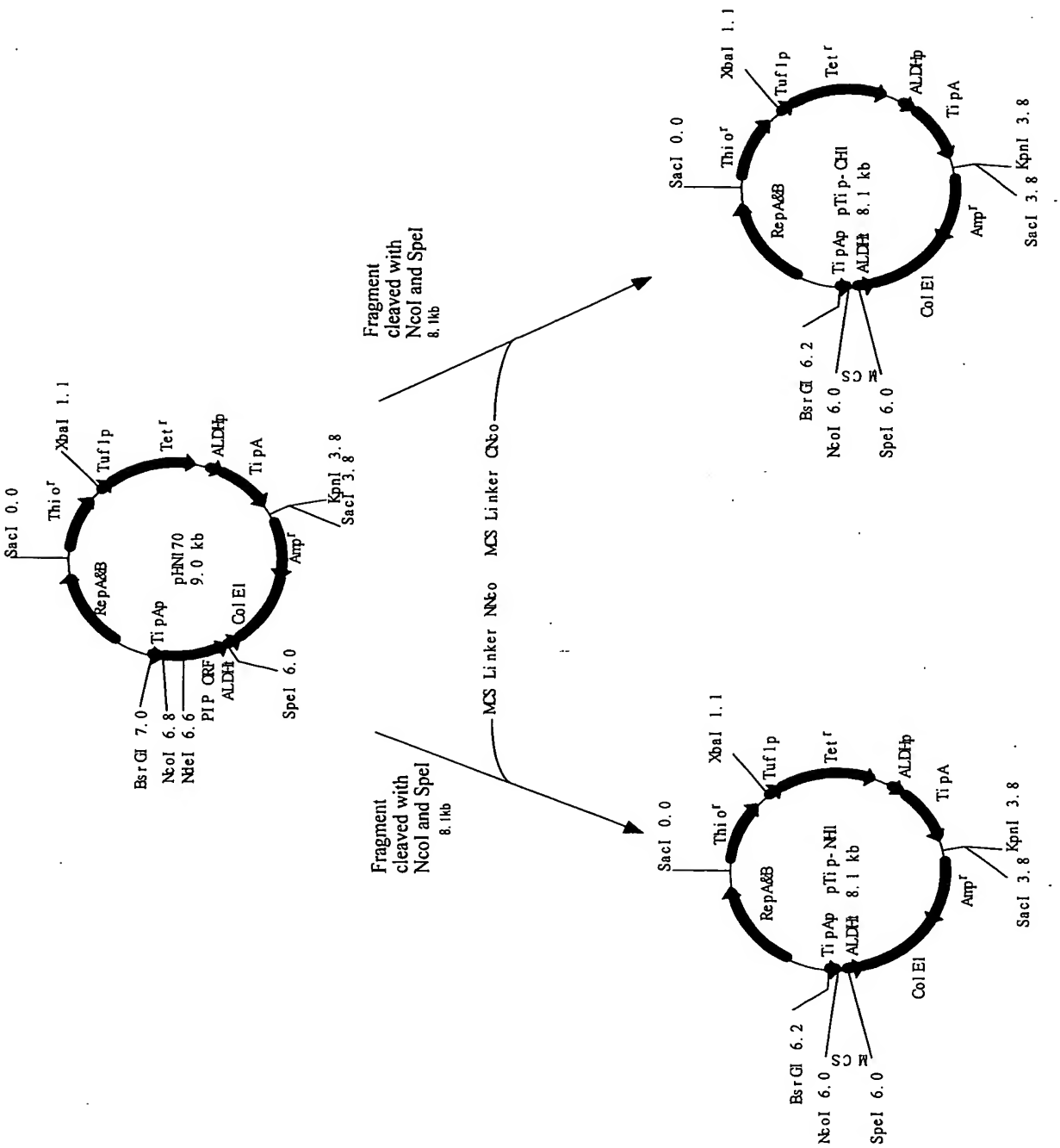


Fig. 7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RepA&B
ColE1
TipAp
TipA
ALDHp
Tetr
Tuf1p
XbaI 1.1
Thior
SacI 0.0
KpnI 3.8
SacI 3.8
Ampr
SpeI 6.0
BsrGI 7.0
NcoI 6.8
NdeI 6.6
PIP ORF
ALDH
MCS Linker NNco
MCS Linker CNco
Fragment cleaved with NcoI and SpeI 8.1kb
Fragment cleaved with NcoI and SpeI 8.1kb
pTip-CHI
8.1 kb
pTip-NHI
8.1 kb
BsrGI 6.2
NcoI 6.0
MCS
SpeI 6.0

Fig. 7 (continued)

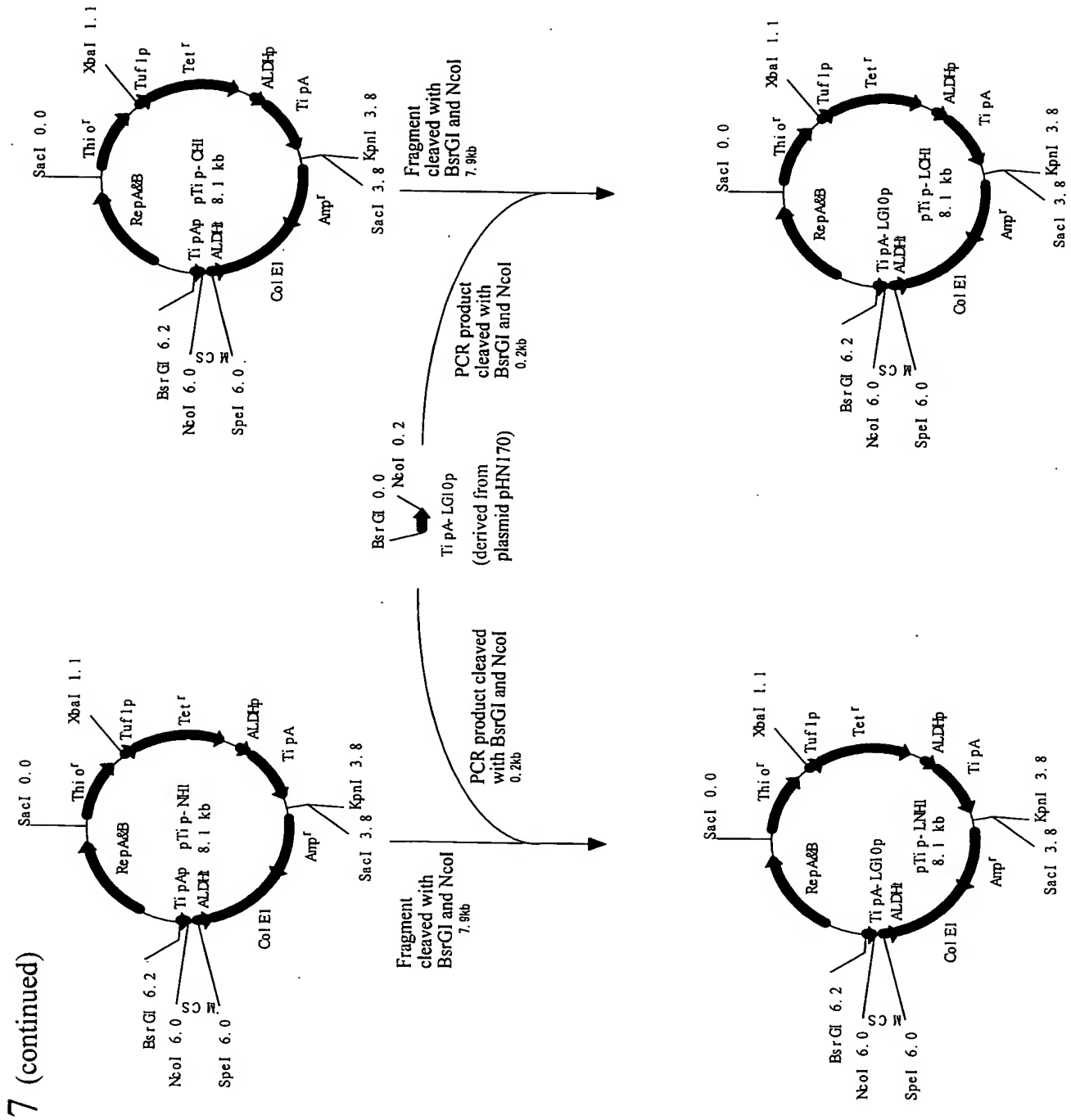


Fig. 8

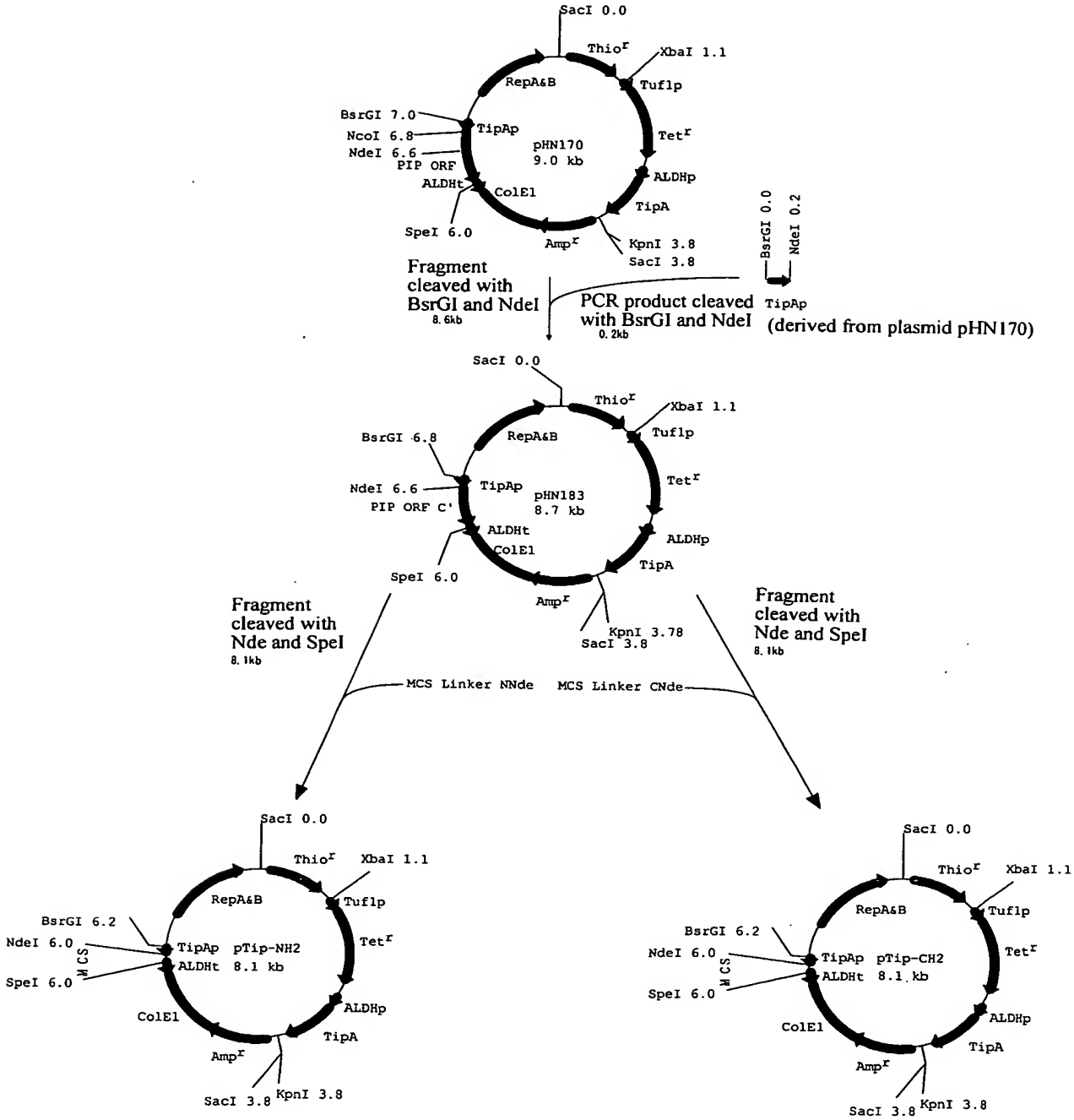

SacI 0.0
Thio^r
XbaI 1.1
RepA&B
TufIp
BsrGI 7.0
TipAp
Tet^r
NcoI 6.8
NdeI 6.6
PIP ORF
pHN170
9.0 kb
ALDHt
ALDHp
ColE1
TipA
SpeI 6.0
Amp^r
KpnI 3.8
SacI 3.8
BsrGI 0.0
NdeI 0.2
Fragment cleaved with BsrGI and NdeI
8.6kb
PCR product cleaved with BsrGI and NdeI
0.2kb
TipAp
(derived from plasmid pHN170)
SacI 0.0
Thio^r
XbaI 1.1
BsrGI 6.8
RepA&B
TufIp
NdeI 6.6
TipAp
Tet^r
PIP ORF C'
pHN183
8.7 kb
ALDHt
ColE1
ALDHp
TipA
SpeI 6.0
Amp^r
KpnI 3.78
SacI 3.8
Fragment cleaved with Nde and SpeI
8.1kb
Fragment cleaved with Nde and SpeI
8.1kb
MCS Linker NNde
MCS Linker CNde
SacI 0.0
Thio^r
XbaI 1.1
RepA&B
TufIp
BsrGI 6.2
NdeI 6.0
TipAp
pTip-NH2
Tet^r
MCS
ALDHt
8.1 kb
SpeI 6.0
ALDHp
ColE1
TipA
Amp^r
SacI 3.8
KpnI 3.8
SacI 0.0
Thio^r
XbaI 1.1
RepA&B
TufIp
BsrGI 6.2
NdeI 6.0
TipAp
pTip-CH2
Tet^r
MCS
ALDHt
8.1 kb
SpeI 6.0
ALDHp
ColE1
TipA
Amp^r
SacI 3.8
KpnI 3.8

Fig. 8 (continued)

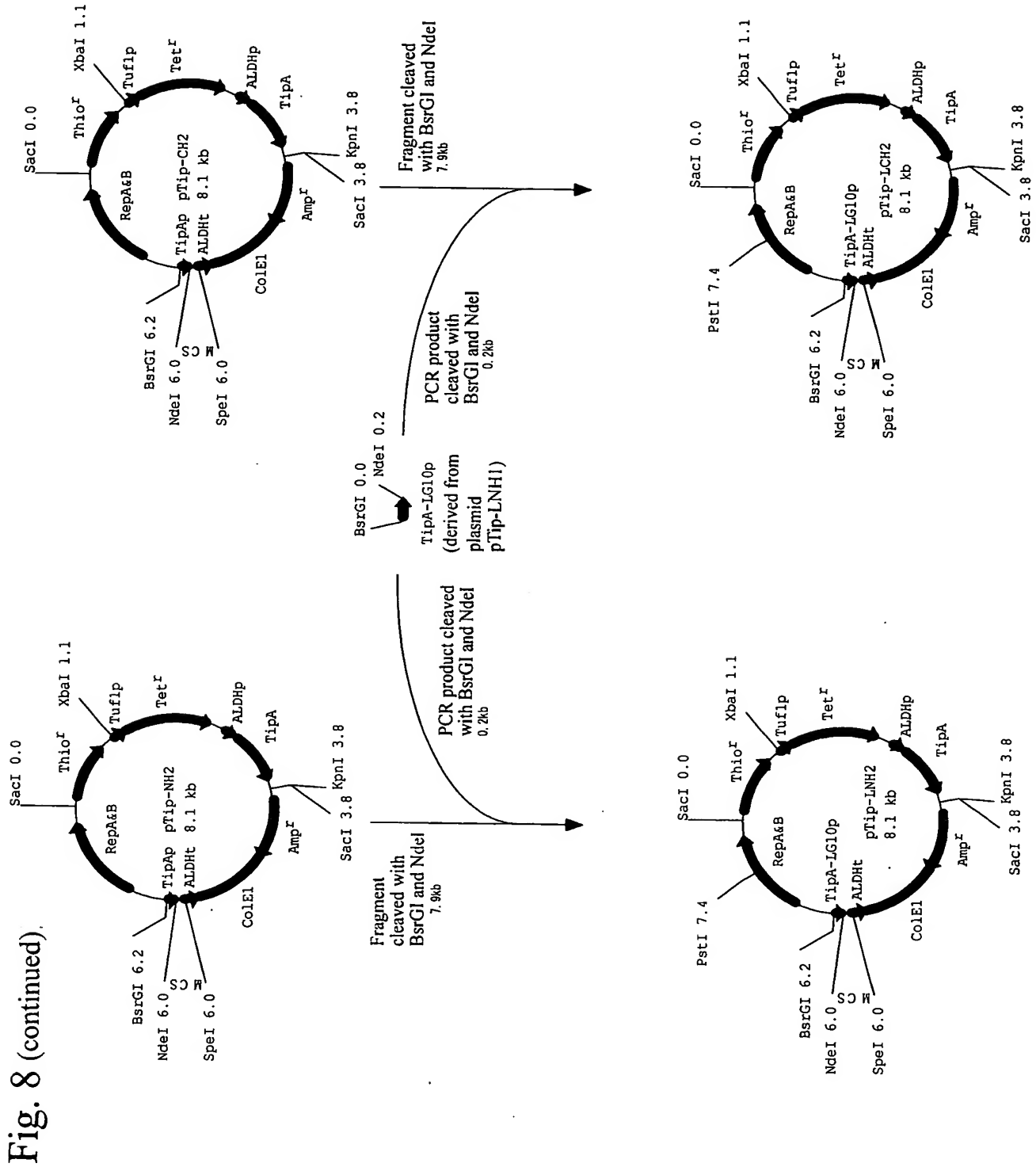
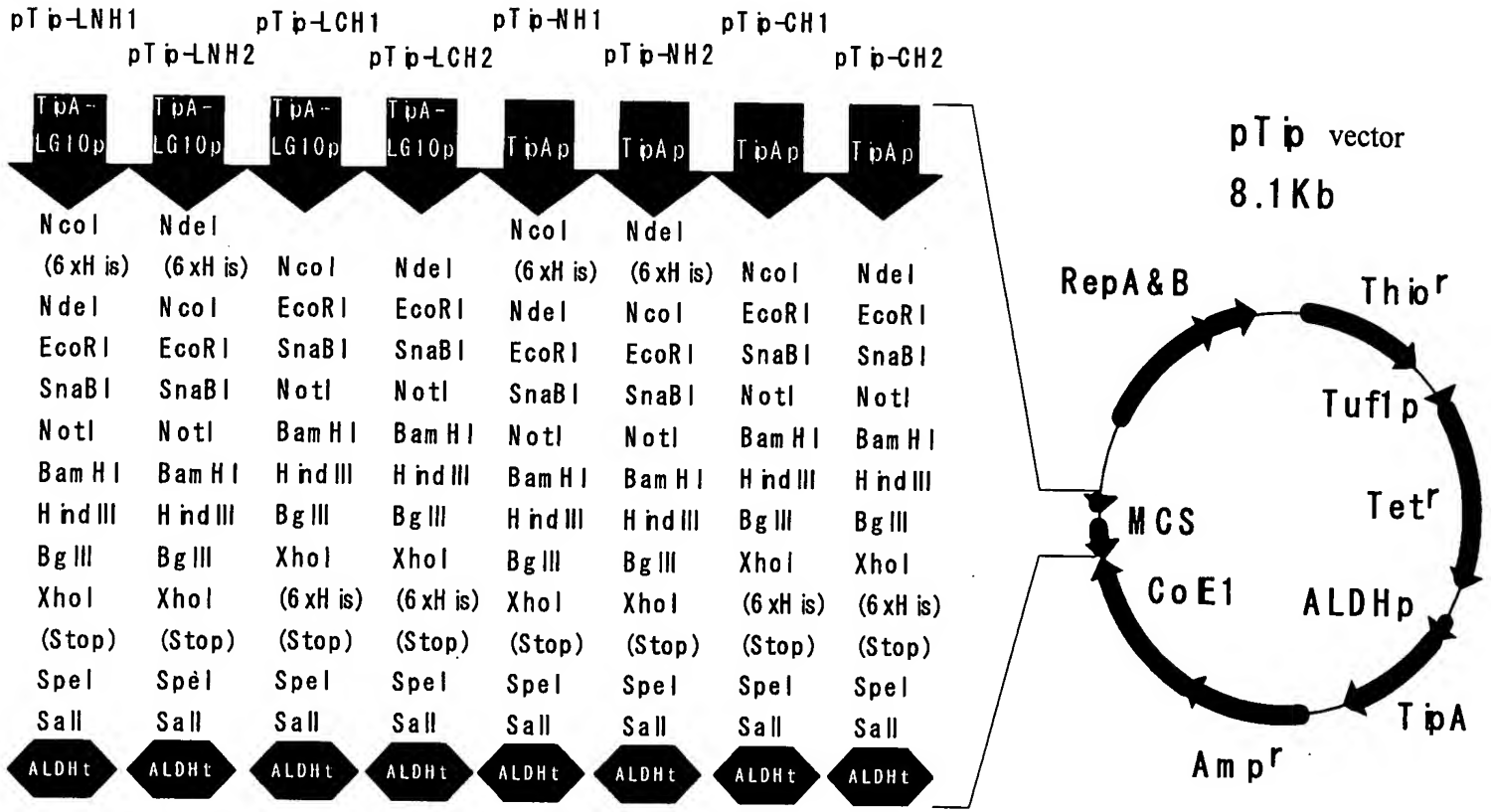


Fig. 9a



Thiostrepton induction system

Thio^r= confers thiostrepton resistance to *R.erythropolis*

ALDHp = promoter which constitutively produces TipA protein

TipA = encodes a TipA protein

TipAp = TipA promoter

TipA-LG10p = improved TipA promoter

ALDHt = transcription termination sequence

Regions necessary for the autonomous replication of a plasmid

CoIE1 = for *E. coli*

RepA&B = for *R.erythropolis*

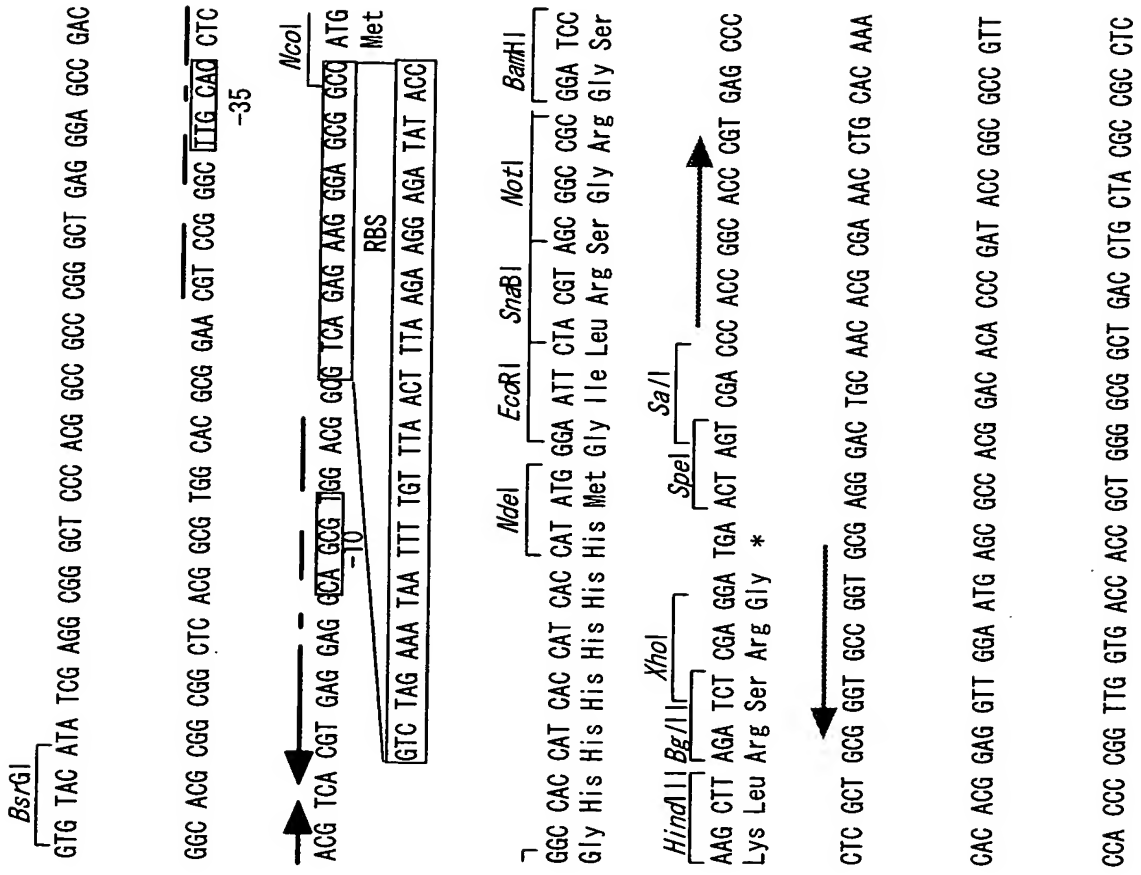
Antibiotic resistance marker

Tuf1p-Tet^r = transformation marker for *R.erythropolis*

Amp^r = transformation marker for *E. coli*

Title: PROCESS FOR PRODUCING RECOMBINANT PROTEIN IN BACTERIUM BELONGING TO THE GENUS RHODOCOCCUS
Inventor(s): Nobutaka NAKASHIMA, et al.
DOCKET NO.: 081356-0253

10/553979


BsrGI
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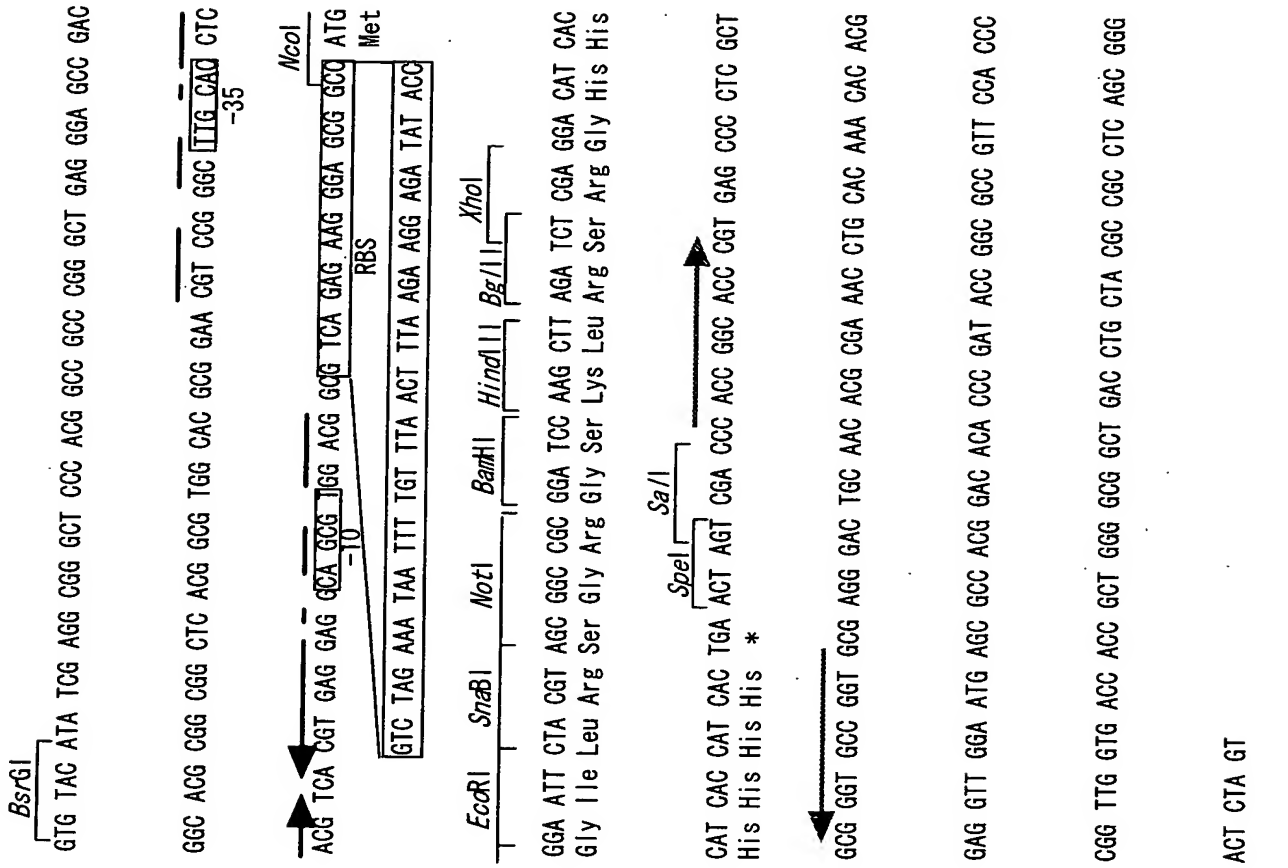
GGC ACG CGG CGG CTC ACG GCG TGG CAC GCG GAA CGT CCG GGC TTG CAC CTC
-35
NcoI
ACG TCA CGT GAG GAG GCA GCG TGG ACG GCG TCA GAG AAG GGA GCG GCC ATG
-10
Met
RBS
GTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT ACC
NdeI
EcoRI
SnaBI
NotI
BamHI
GGC CAC CAT CAC CAT CAC CAT ATG GGA ATT CTA CGT AGC GGC CGC GGA TCC
Gly His His His His His His Met Gly Ile Leu Arg Ser Gly Arg Gly Ser
HindIII
BglII
XhoI
SpeI
SalI
AAG CTT AGA TCT CGA GGA TGA ACT AGT CGA CCC ACC GGC ACC CGT GAG CCC
Lys Leu Arg Ser Arg Gly *
CTC GCT GCG GGT GCC GGT GCG AGG GAC TGC AAC ACG CGA AAC CTG CAC AAA
CAC ACG GAG GTT GGA ATG AGC GCC ACG GAC ACA CCC GAT ACC GGC GCC GTT
CCA CCC CGG TTG GTG ACC ACC GCT GGG GCG GCT GAC CTG CTA CGC CGC CTC
AGC GGG ACT CTA GT

Fig. 9b

Fig. 9c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GGC ACG CGG CGG CTC ACG GCG TGG CAC GCG GAA CGT CCG GGC TTG CAC CTC
-35
NcoI
ACG TCA CGT GAG GAG GCA GCG TGG ACG GCG TCA GAG AAG GGA GCG GCG ATG
-10
RBS
Met
GTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT ACC
EcoRI
SnaBI
NotI
BamHI
HindIII
BglII
XhoI
GGA ATT CTA CGT AGC GGC CGC GGA TCC AAG CTT AGA TCT CGA GGA CAT CAC
Gly Ile Leu Arg Ser Gly Arg Gly Ser Lys Leu Arg Ser Arg Gly His His
SpeI
SalI
CAT CAC CAT CAC TGA ACT AGT CGA CCC ACC GGC ACC CGT GAG CCC CTC GCT
His His His His *
GCG GGT GCC GGT GCG AGG GAC TGC AAC ACG CGA AAC CTG CAC AAA CAC ACG
GAG GTT GGA ATG AGC GCC ACG GAC ACA CCC GAT ACC GGC GCC GTT CCA CCC
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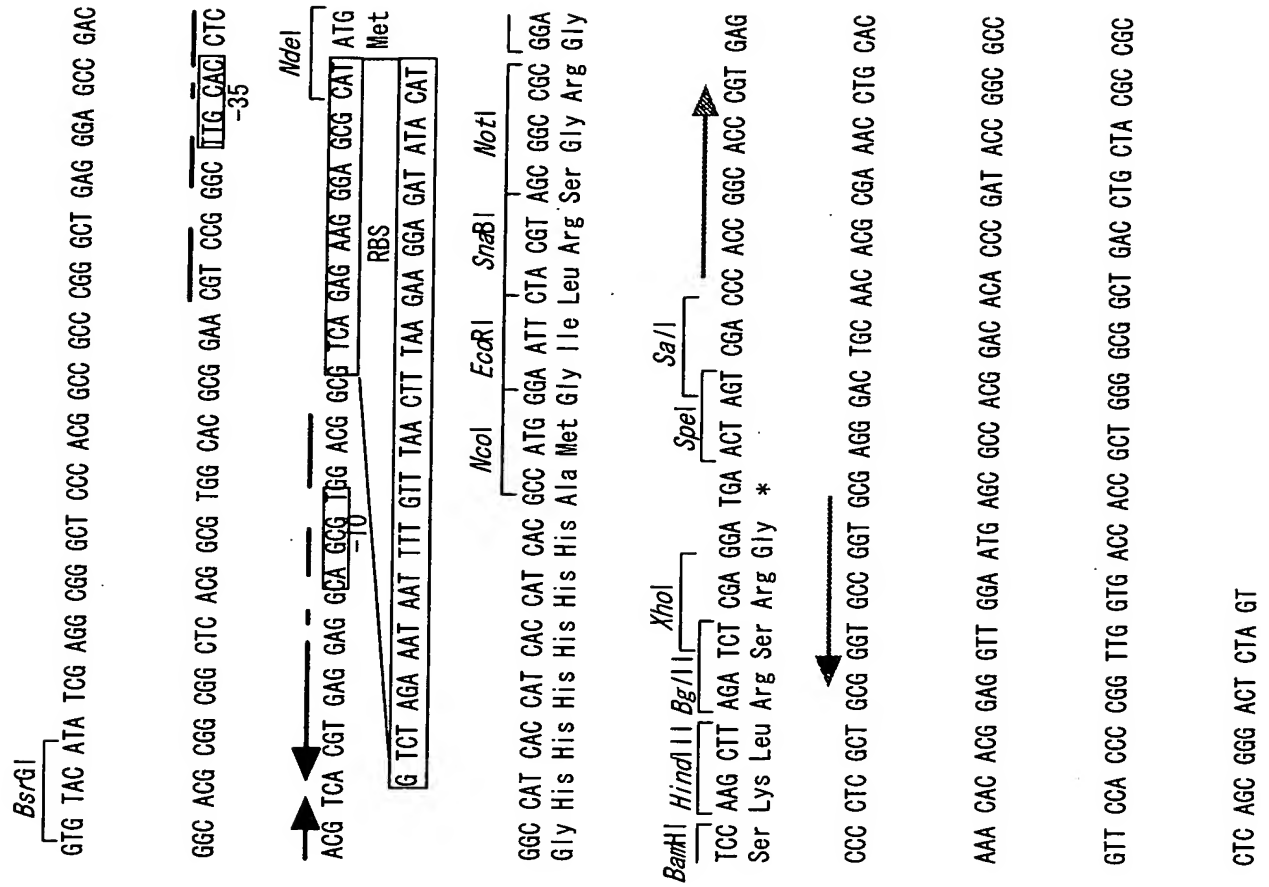
ACT CTA GT

Fig. 9d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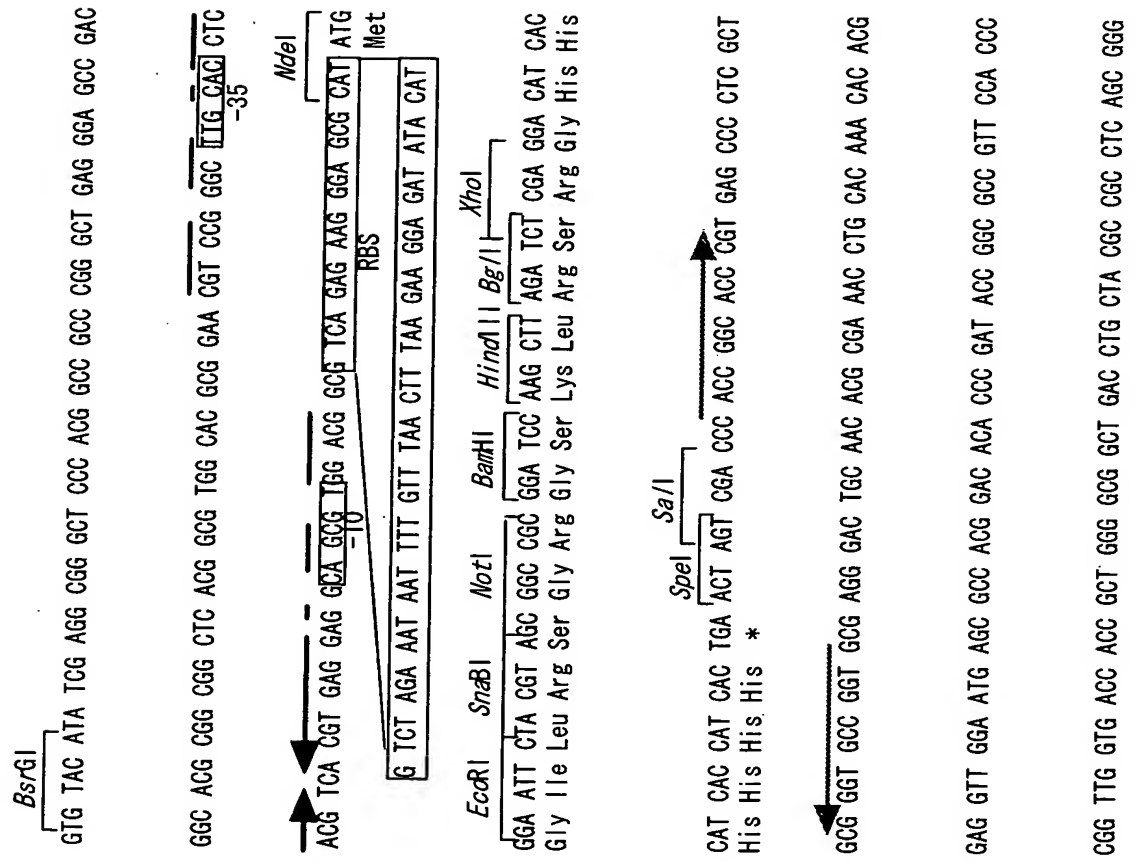
GGC ACG CGG CGG CTC ACG GCG TGG CAC GCG GAA CGT CCG GGC TTG CAC CTC
-35
NdeI
ACG TCA CGT GAG GAG GCA GCG TGG ACG GCG TCA GAG AAG GGA GCG CAT ATG
-10
Met
RBS
G TCT AGA AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT
NcoI EcoRI SnaBI NotI
GGC CAT CAC CAT CAC CAT CAC GCC ATG GGA ATT CTA CGT AGC GGC CGC GGA
Gly His His His His His Ala Met Gly Ile Leu Arg Ser Gly Arg Gly
XhoI
BamHI HindIII BglII SpeI SalI
TCC AAG CTT AGA TCT CGA GGA TGA ACT AGT CGA CCC ACC GGC ACC CGT GAG
Ser Lys Leu Arg Ser Arg Gly *
CCC CTC GCT GCG GGT GCC GGT GCG AGG GAC TGC AAC ACG CGA AAC CTG CAC
AAA CAC ACG GAG GTT GGA ATG AGC GCC ACG GAC ACA CCC GAT ACC GGC GCC
GTT CCA CCC CGG TTG GTG ACC ACC GCT GGG GCG GCT GAC CTG CTA CGC CGC
CTC AGC GGG ACT CTA GT

Fig. 9e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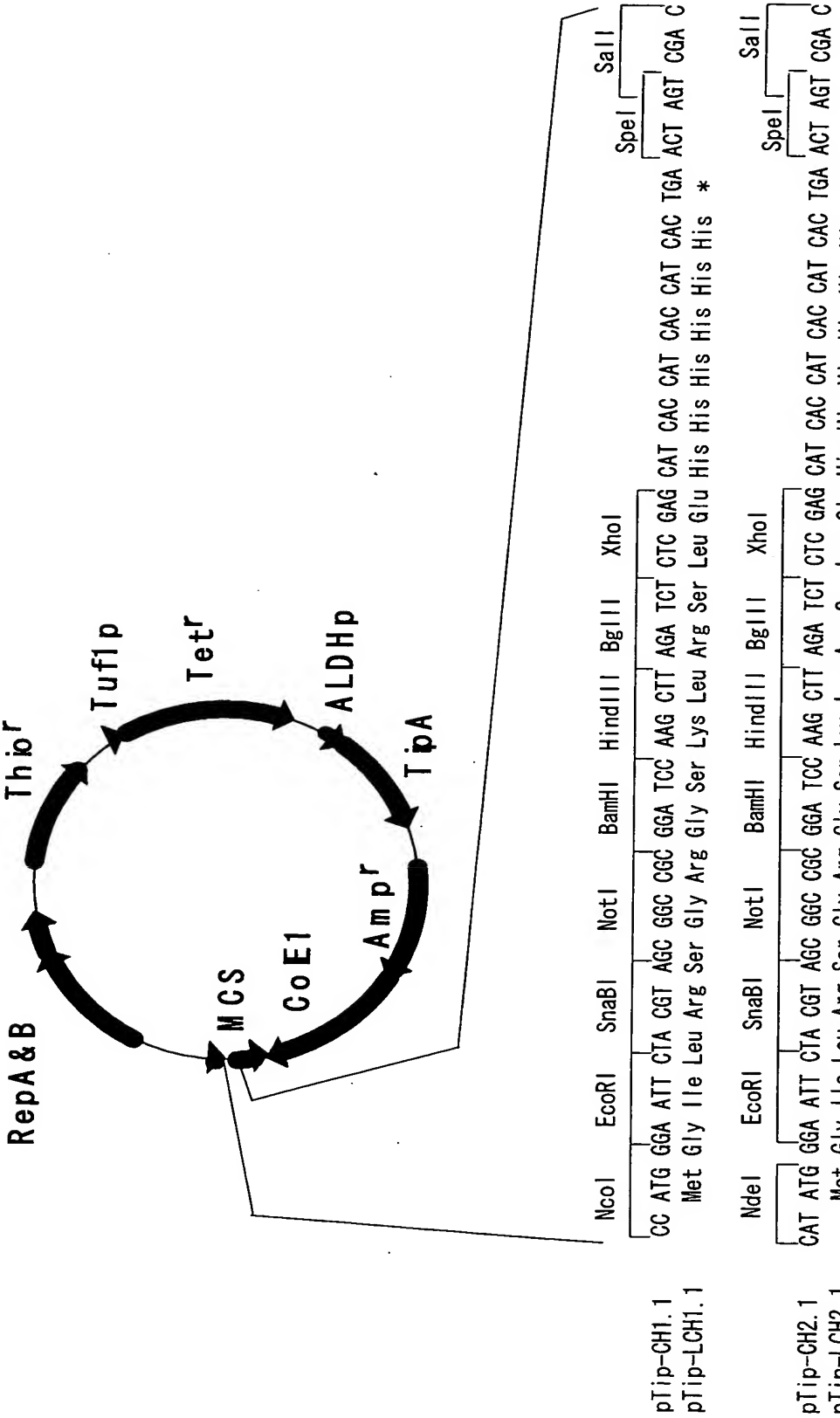
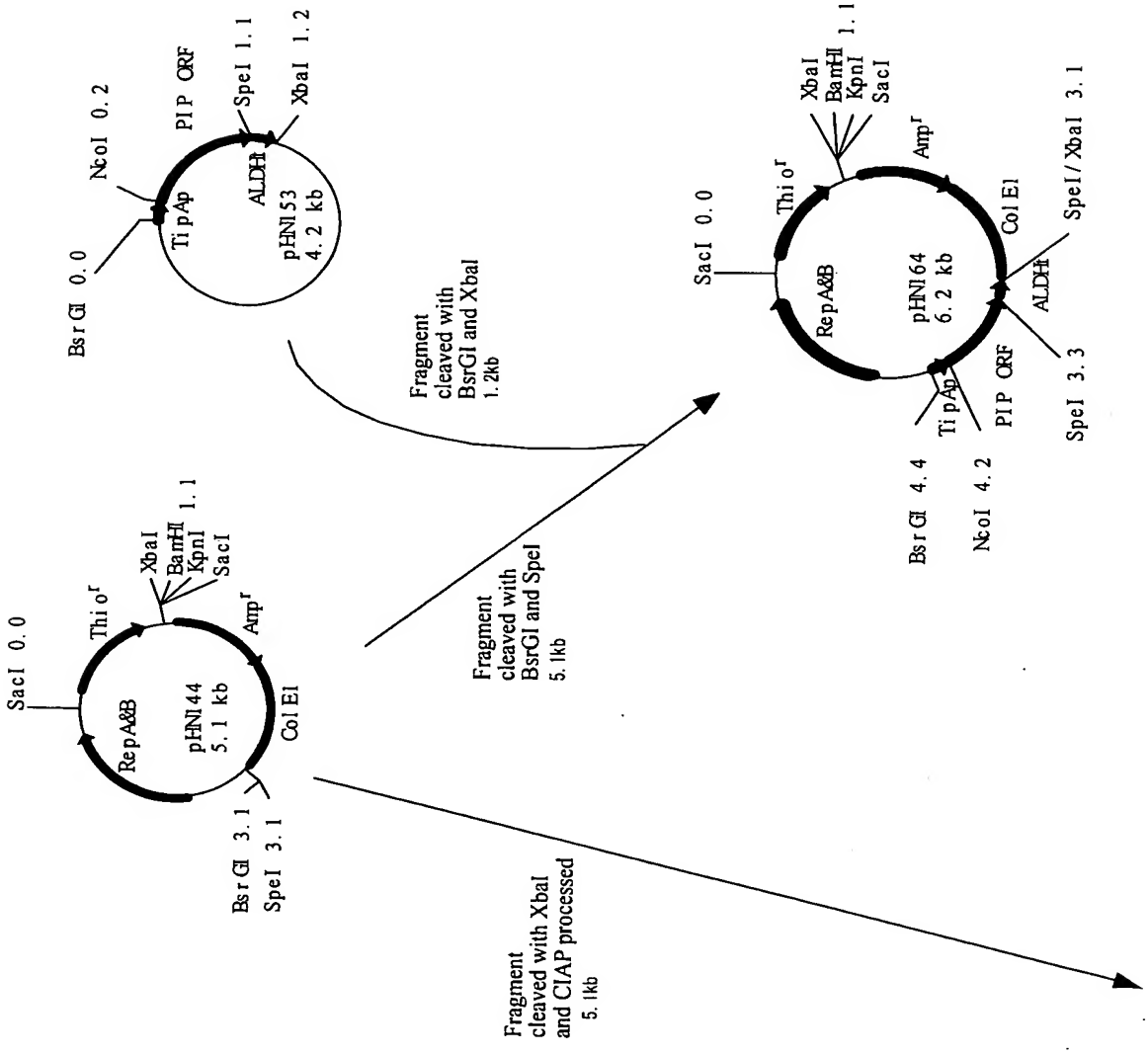
GGC ACG CGG CGG CTC ACG GCG TGG CAC GCG GAA CGT CCG GGC TTG CAC CTC
-35
NdeI
ACG TCA CGT GAG GAG GCA GCG TGG ACG GCG TCA GAG AAG GGA GCG CAT ATG
-10
Met
RBS
G TCT AGA AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT
EcoRI SnaBI NotI BamHI HindIII BglII XhoI
GGA ATT CTA CGT AGC GGC CGC GGA TCC AAG CTT AGA TCT CGA GGA CAT CAC
Gly Ile Leu Arg Ser Gly Arg Gly Ser Lys Leu Arg Ser Arg Gly His His
SpeI SalI
CAT CAC CAT CAC TGA ACT AGT CGA CCC ACC GGC ACC CGT GAG CCC CTC GCT
His His His His *
GCG GGT GCC GGT GCG AGG GAC TGC AAC ACG CGA AAC CTG CAC AAA CAC ACG
GAG GTT GGA ATG AGC GCC ACG GAC ACA CCC GAT ACC GGC GCC GTT CCA CCC
CGG TTG GTG ACC ACC GCT GGG GCG GCT GAC CTG CTA CGC CGC CTC AGC GGG
ACT CTA GT

Fig. 10


Thio^r
Tuf1p
Tet^r
ALDHp
TipA
Amp^r
CoE1
MCS
RepA&B
pTip-CH1.1
pTip-LCH1.1
NcoI EcoRI SnaBI NotI BamHI HindIII BglII XhoI
CC ATG GGA ATT CTA CGT AGC GGC CGC GGA TCC AAG CTT AGA TCT CTC GAG CAT CAC CAT CAC CAT CAC TGA ACT AGT CGA C
Met Gly Ile Leu Arg Ser Gly Arg Gly Ser Lys Leu Arg Ser Leu Glu His His His His His His * SpeI SalI
pTip-CH2.1
pTip-LCH2.1
NdeI EcoRI SnaBI NotI BamHI HindIII BglII XhoI
CAT ATG GGA ATT CTA CGT AGC GGC CGC GGA TCC AAG CTT AGA TCT CTC GAG CAT CAC CAT CAC CAT CAC TGA ACT AGT CGA C
Met Gly Ile Leu Arg Ser Gly Arg Gly Ser Lys Leu Arg Ser Leu Glu His His His His His His * SpeI SalI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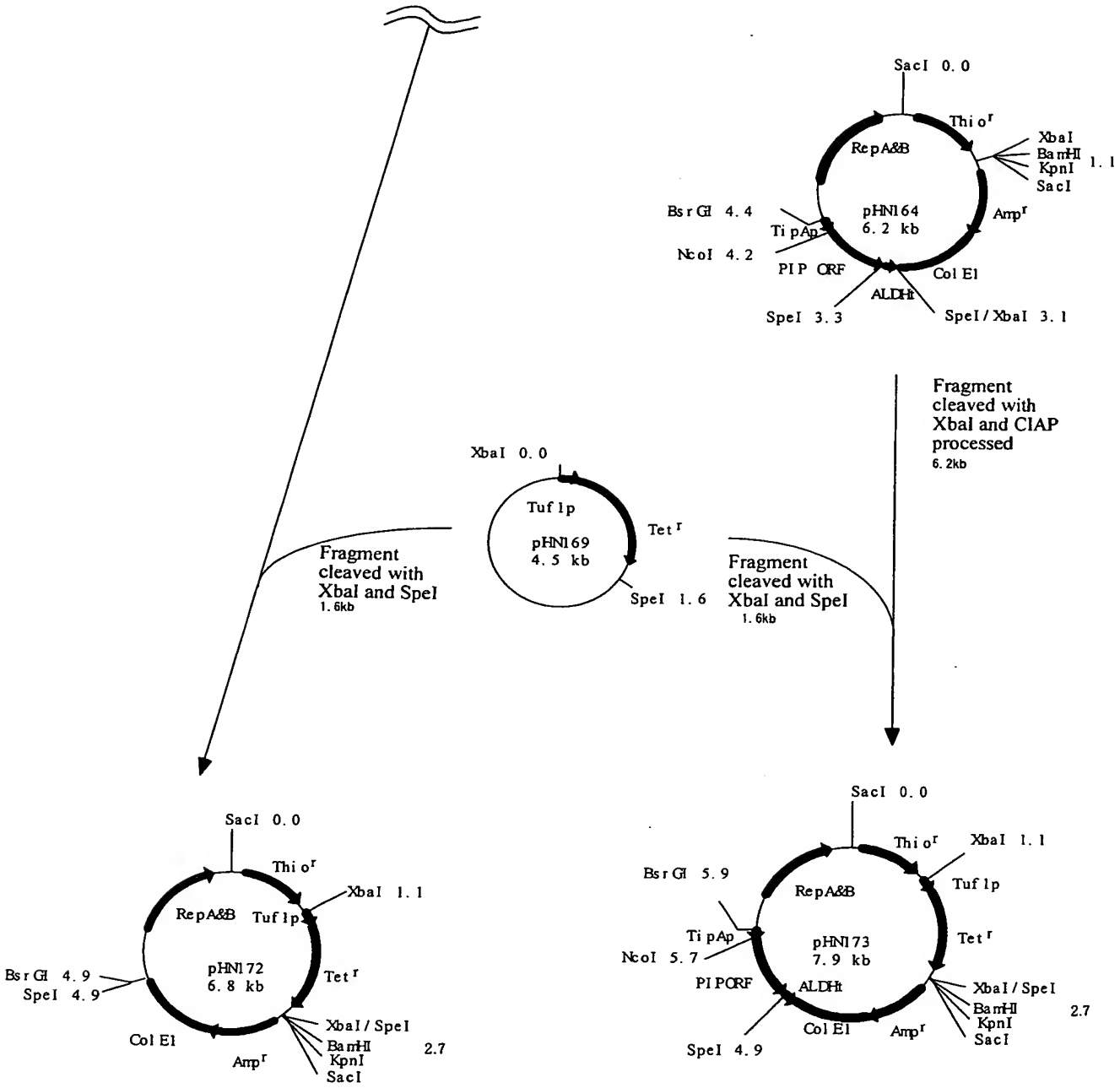
NcoI 0.2
TipAp
PIP ORF
SpeI 1.1
ALDH
XbaI 1.2
pHN153
4.2 kb
Fragment
cleaved with
BsrGI and XbaI
1.2kb
SacI 0.0
RepA&B
Thio^r
XbaI
BamHI
KpnI
SacI
1.1
Amp^r
pHN144
5.1 kb
ColE1
BsrGI 3.1
SpeI 3.1
Fragment
cleaved with
BsrGI and SpeI
5.1kb
Fragment
cleaved with XbaI
and CIAP processed
5.1kb
SacI 0.0
Thio^r
XbaI
BamHI
KpnI
SacI
1.1
Amp^r
RepA&B
pHN164
6.2 kb
ColE1
SpeI/XbaI 3.1
ALDH
SpeI 3.3
PIP ORF
TipAp
BsrGI 4.4
NcoI 4.2

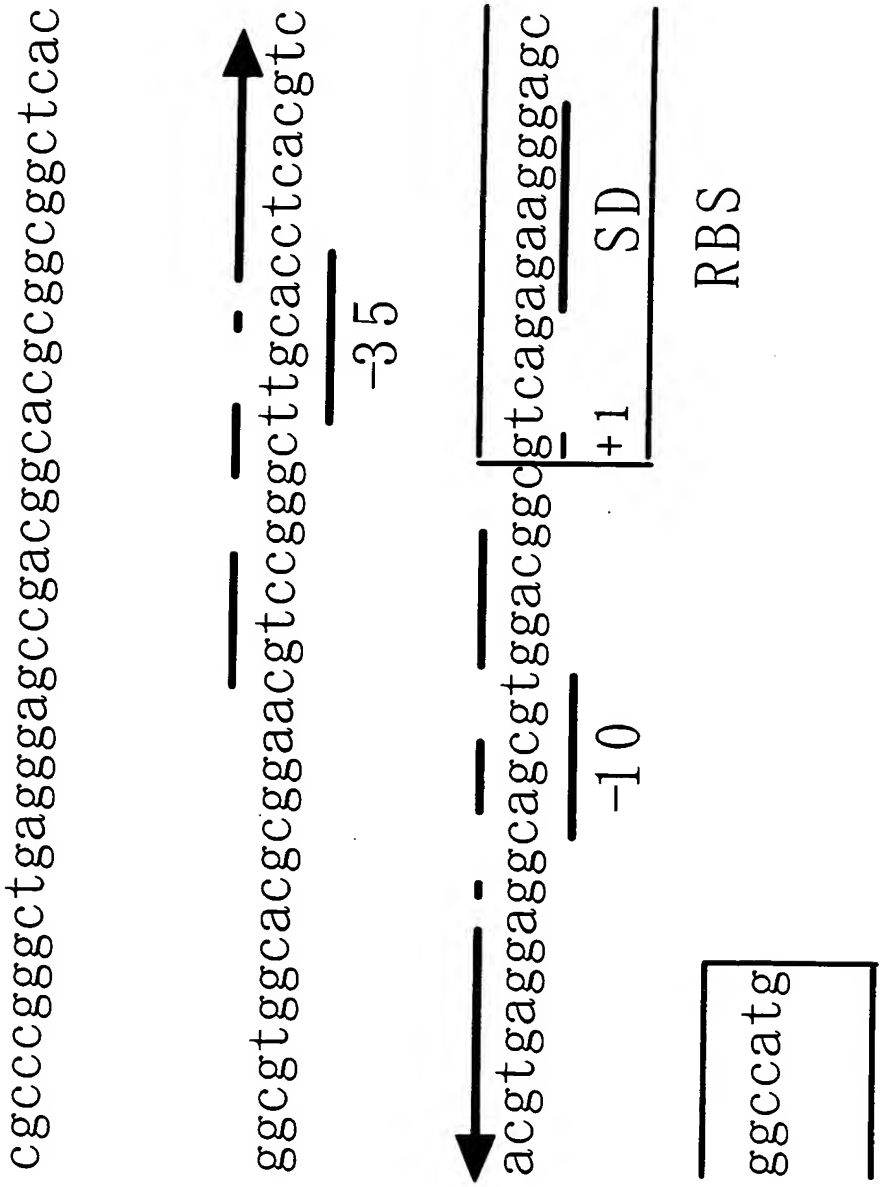
Fig. 11

Fig. 11 (continued)



Title: PROCESS FOR PRODUCING RECOMBINANT PROTEIN IN BACTERIUM BELONGING TO THE GENUS RHODOCOCCUS
Inventor(s): Nobutaka NAKASHIMA, et al.
DOCKET NO.: 081356-0253

Fig. 12




WT RBS
g tca ggg aag gga ggg gcc atg
SD
LG10 RBS
gtc tag aaa taa ttt tgt tta act tta agg agg tat acc atg
SD

Fig. 13

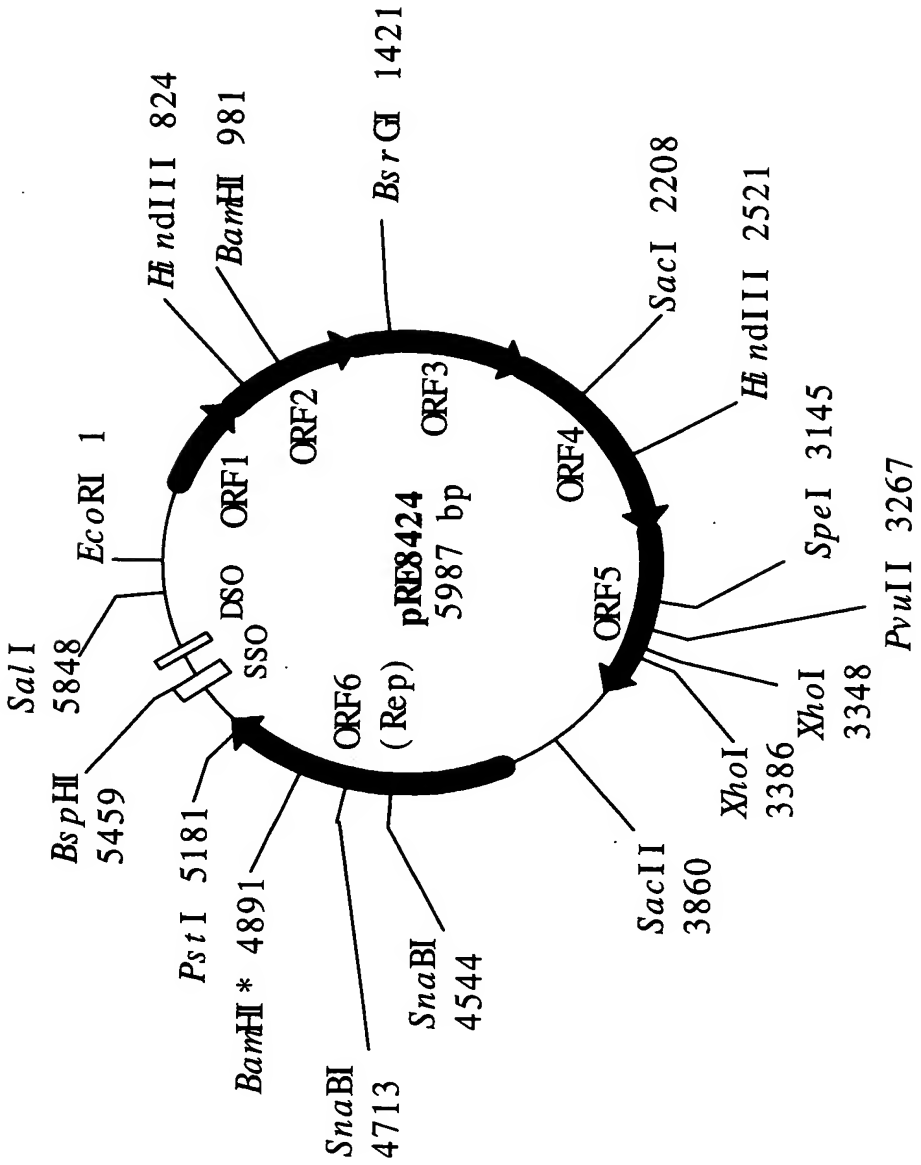

pRE8424
5987 bp
ORF1
ORF2
ORF3
ORF4
ORF5
ORF6
(Rep)
DSO
SSO
EcoRI 1
HindIII 824
BamHI 981
BsrGI 1421
SacI 2208
HindIII 2521
SpeI 3145
PvuII 3267
XhoI 3348
XhoI 3386
SacII 3860
SnaBI 4544
SnaBI 4713
BamHI * 4891
PstI 5181
BspHI 5459
SalI 5848

Fig. 14

Fig. 15

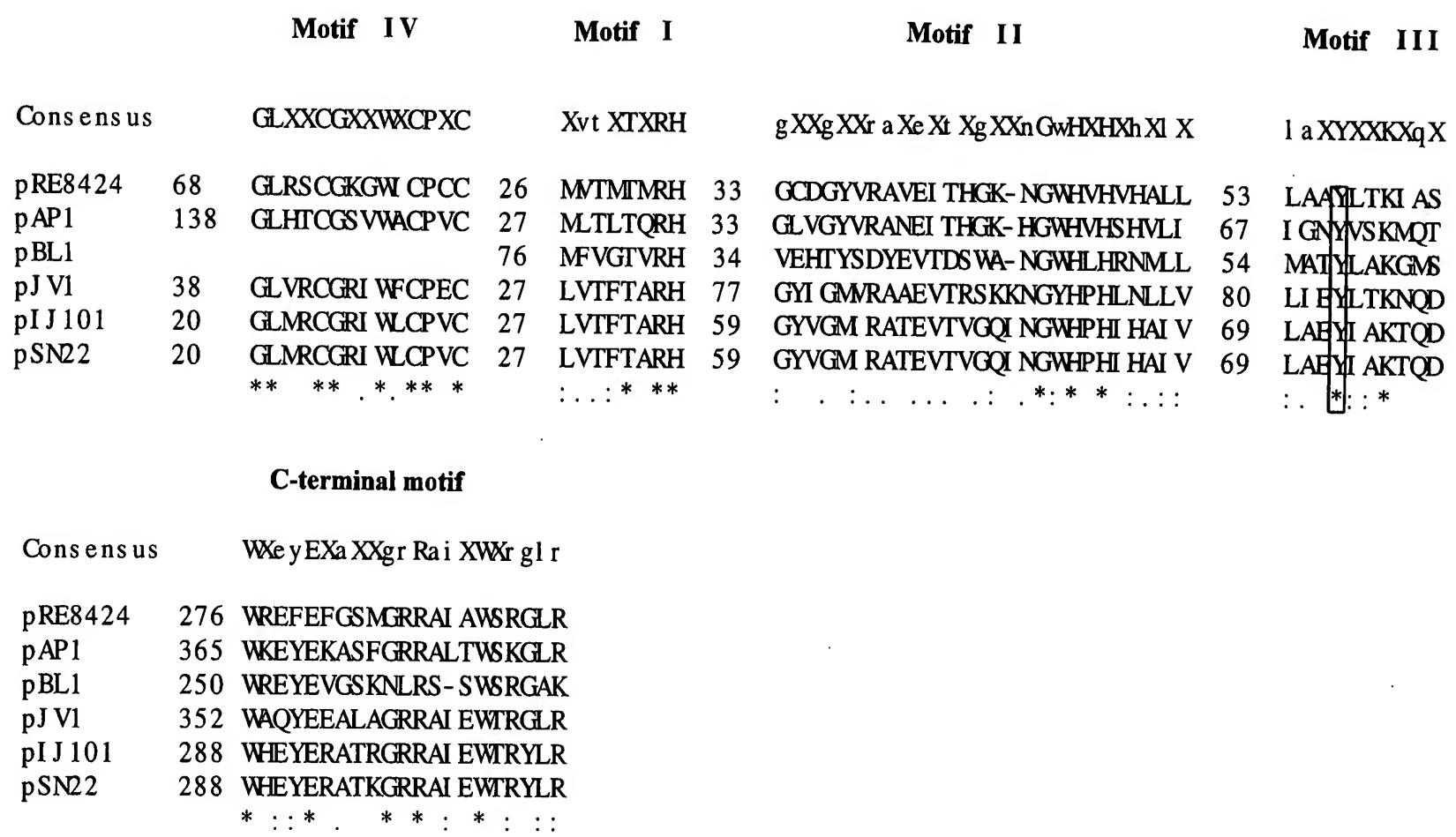


Fig. 16

pRE8424 5705 CGAGCGAAGCG-GA-CGCCCT---AGCTGGGGGAG-
pAP1 2378 CAGGTATGC-G-GA-AAACTTT--AGCAACAA----
pBL1 1314 GAAATAGAA-CTGA-ACACGTCTAAGCAACCGCA--
pJV1 3375 CTGGCAAAAAGCGA--CGCCT---AGCTAAAGGGTT
pIJ101 1346 GAGCCAAAA-CCGA-ACACGTT--CGCAAAGAAA--
pSN22 7805 GACCCAAAACCTCTCGCCCGTT--CGCAAAGAAA--
Nicking site
DSO

Fig. 17

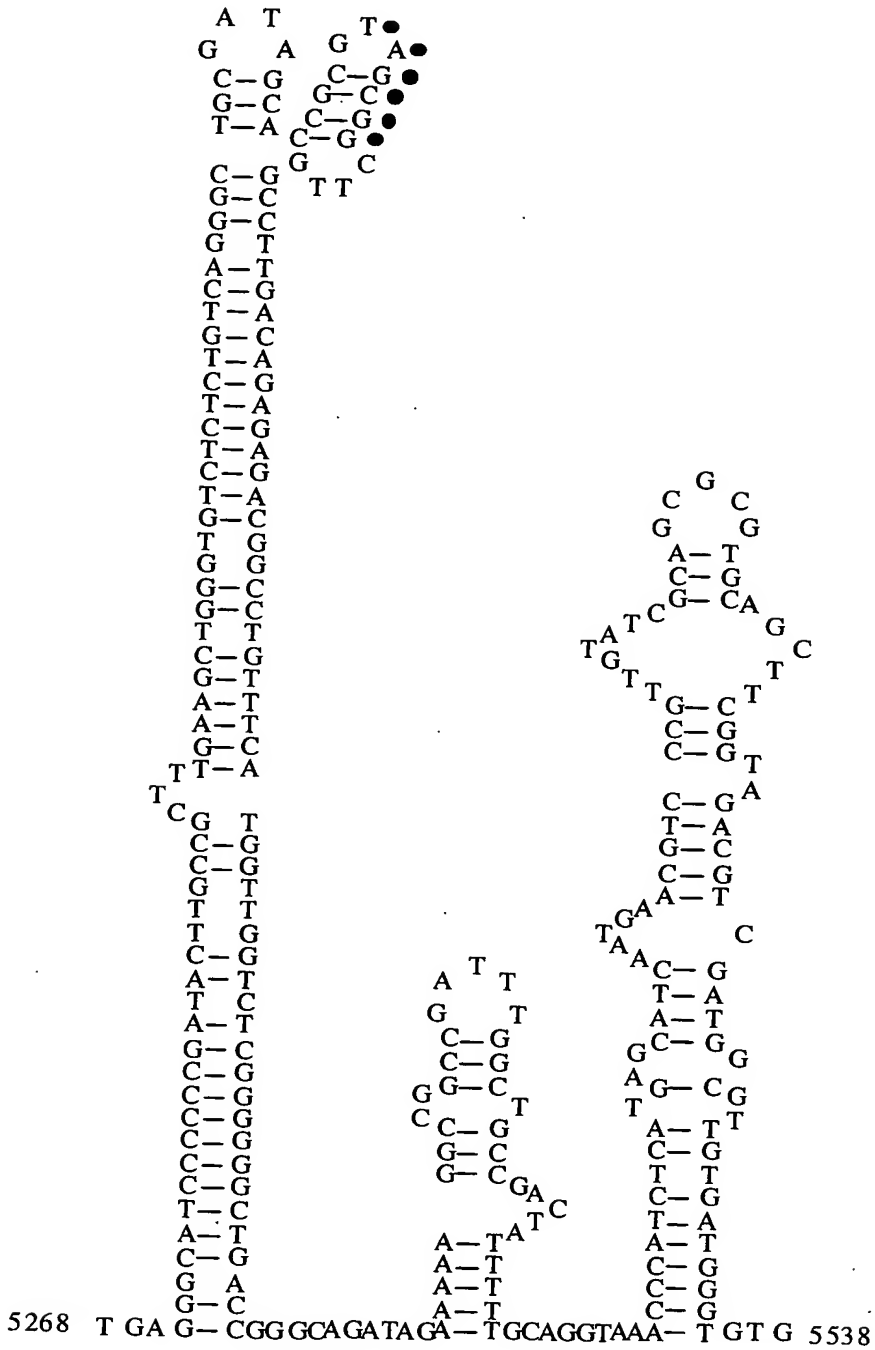
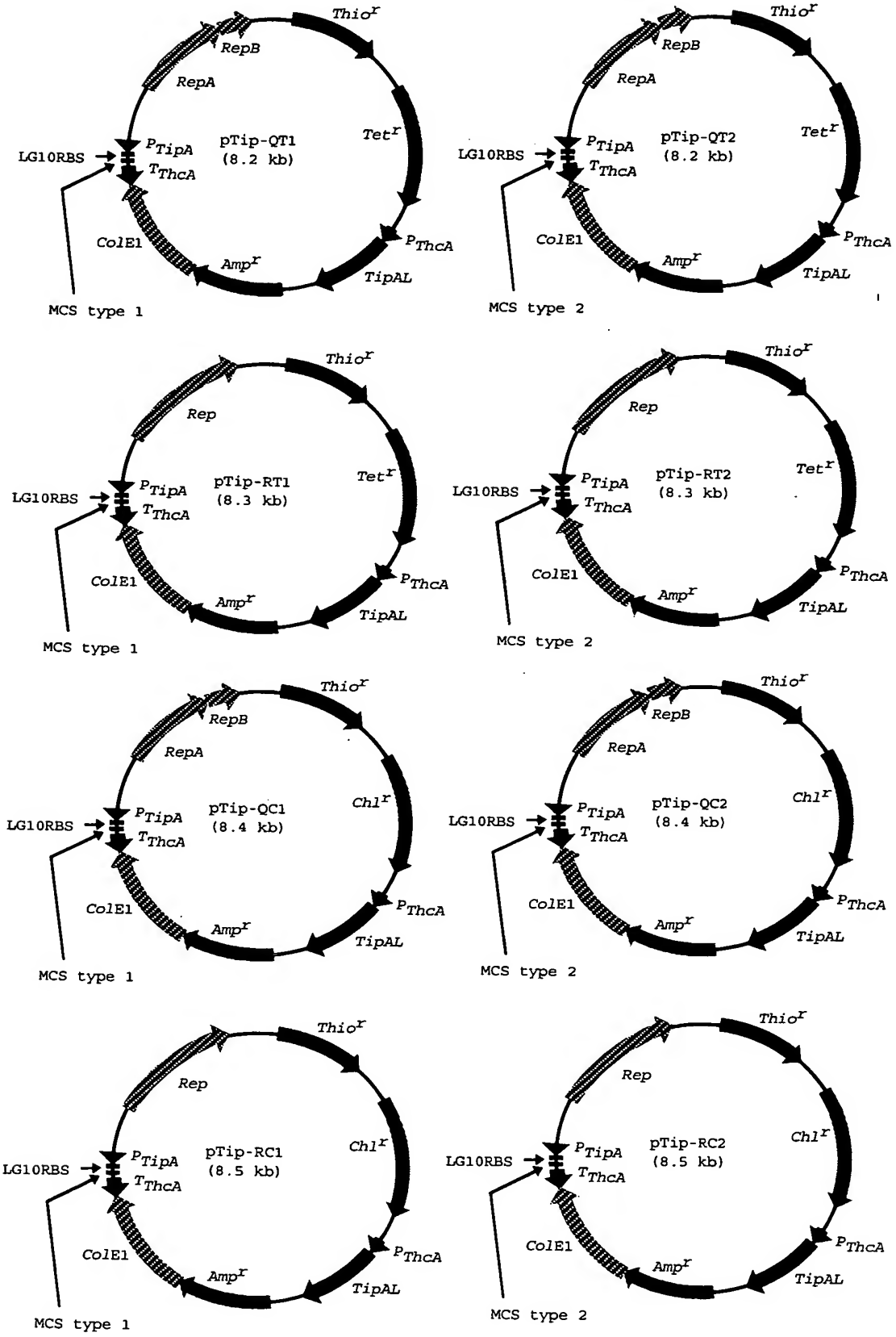


Fig. 18-1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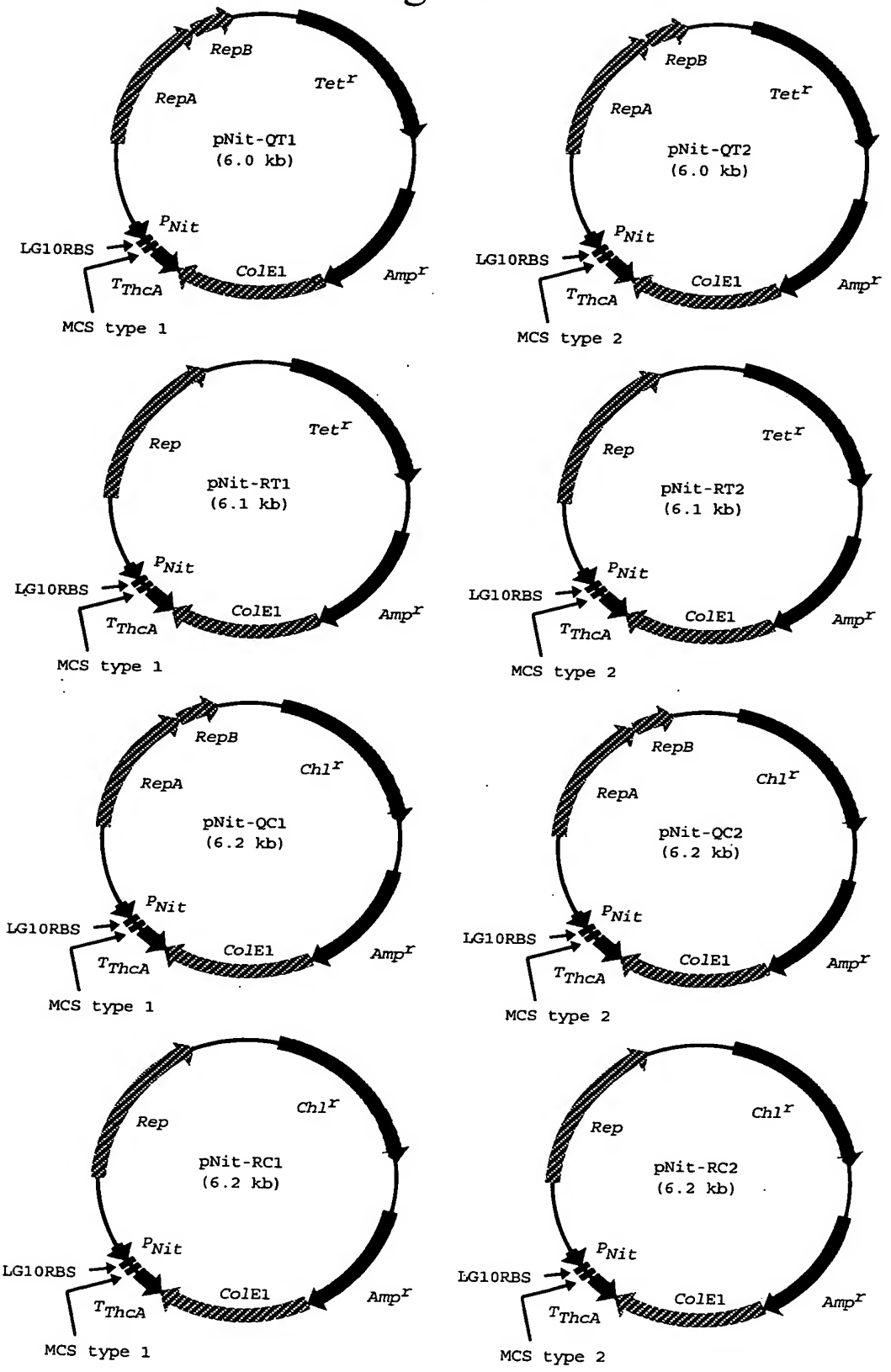
(8.2 kb)
pTip-QT2
(8.2 kb)
pTip-RT1
(8.3 kb)
pTip-RT2
(8.3 kb)
pTip-QC1
(8.4 kb)
pTip-QC2
(8.4 kb)
pTip-RC1
(8.5 kb)
pTip-RC2
(8.5 kb)
Thio^r
RepB
RepA
Rep
Tet^r
Chl^r
P_{TipA}
T_{ThcA}
LG10RBS
ColE1
Amp^r
P_{ThcA}
TipAL
MCS type 1
MCS type 2

Fig. 18-2


RepB
Tet^r
RepA
pNit-QT1
(6.0 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 1
RepB
Tet^r
RepA
pNit-QT2
(6.0 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 2
Rep
Tet^r
pNit-RT1
(6.1 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 1
Rep
Tet^r
pNit-RT2
(6.1 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 2
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Chl^r
RepA
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(6.2 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 1
RepB
Chl^r
RepA
pNit-QC2
(6.2 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 2
Rep
Chl^r
pNit-RC1
(6.2 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 1
Rep
Chl^r
pNit-RC2
(6.2 kb)
P_Nit
LG10RBS
T_ThcA
ColE1
Amp^r
MCS type 2

Fig. 19

TipA-LG10p or *Nit-LG10p*

*Bsr*GI

TG TAC ATA TCG AGG CGG GCT CCC ACG GCC GCC CGG GCT GAG GGA GCC GAC GGC ACG CGG CGG CTC ACG GCG TGG CAC GCG GAA CGT CCG GGC

TTG CAC CTC ACG TCA CGT GAG GAG GCA GCG TGG ACG GCG TCT AGA AAT AAT TTT GTT TAA CTT TAA GAA GAA GAT ATA

-35

TA TAA T

-10

MCS

Type 1

*Nco*I *Nde*I *Eco*RI *Sna*BI *Not*I *Bam*HI *Hin*dIII *Bgl*II *Xho*I

CC ATG GGC CAC CAT CAC CAT CAC CAT ATG GGA ATT CTA CGT AGC GGC CGC GGA TCC AAG CTT AGA TCT CTC GAG CAT CAC CAT CAC CAT CAC TGA

Met Gly His His His His His His Met Gly Ile Leu Arg Ser Gly Arg Gly Ser Lys Leu Arg Ser Leu Glu His His His His His His *

Type 2

*Nde*I *Nco*I *Eco*RI *Sna*BI *Not*I *Bam*HI *Hin*dIII *Bgl*II *Xho*I

CAT ATG GGC CAT CAC CAT CAC CAT CAC GCC ATG GGA ATT CTA CGT AGC GGC CGC GGA TCC AAG CTT AGA TCT CTC GAG CAT CAC CAT CAC CAT CAC TGA

Met Gly His His His His His His Ala Met Gly Ile Leu Arg Ser Gly Arg Gly Ser Lys Leu Arg Ser Leu Glu His His His His His His *

ALDHt

*Spe*I *Sal*I

ACT AGT CGA CCC ACC GGC ACC CGT GAG CCC CTC GCT GCG GGT GCC GGT GCG AGG GAC TGC AAC ACG CGA AAC CTG CAC AAA CAC ACG GAG GTT

GGA ATG AGC GCC ACG GAC ACA CCC GAT ACC GGC GCC GTT CCA CCC CGG TTG GTG ACC ACC GCT GGG GCG GCT GAC CTG CTA CGC CGC CTC AGC

GGG ACT CTA GT

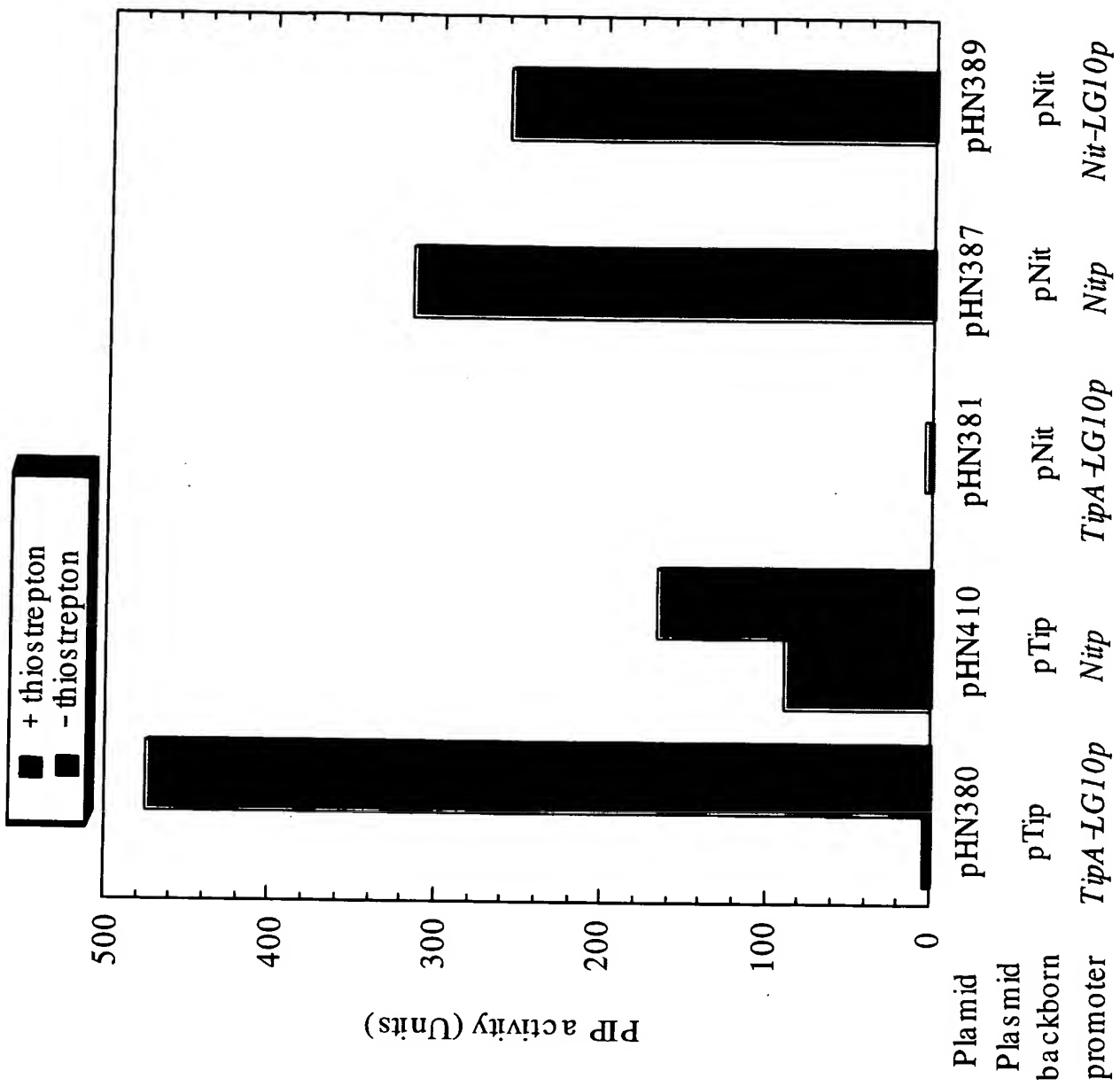
+ thiostrepton
- thiostrepton
PIP activity (Units)
0
100
200
300
400
500
Plamid
Plasmid backborn
promoter
pHN380
pTip
TipA-LG10p
pHN410
pTip
Nitp
pHN381
pNit
TipA-LG10p
pHN387
pNit
Nitp
pHN389
pNit
Nit-LG10p

Fig. 20

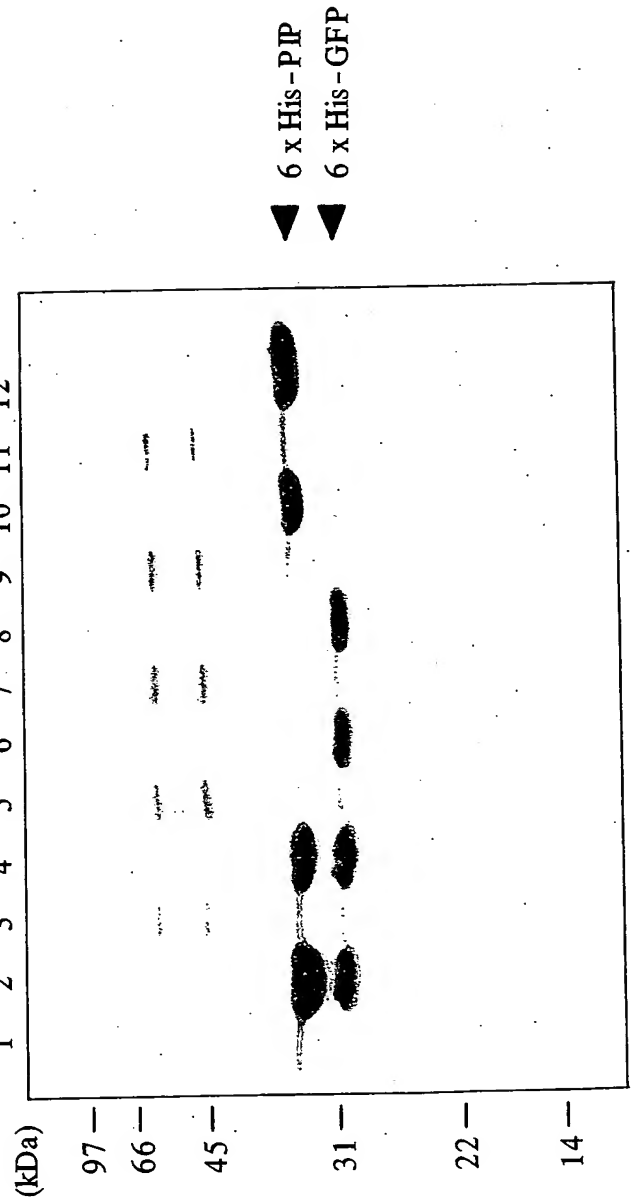

Fig. 21